The Roles of Tumor-suppressive microRNA-181a and *CYFIP1* in Cancerous Proliferation and Invasion of Cutaneous Squamous Cell Carcinoma

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Table of contents

1. Zusammenfassung

Bei Plattenepithelkarzinom der Haut (SCC) handelt es sich um den zweithäufigsten Hautkrebs innerhalb der kaukasischen Bevölkerung. Die Inzidenz dieser Krebserkrankung ist in den letzten Jahrzehnten konstant angestiegen und ist zurzeit, auf Grund dessen nicht zu unterschätzenden Hang zur Metastasierung, für ein Viertel aller hautkrebsassoziierten Todesfälle weltweit verantwortlich. Chronische Sonnenexposition wird als Hauptursache für die SCC Entstehung gesehen. Der darin enthaltene Anteil an UV-Strahlung schädigt die DNA der Keratinozyten direkt und hinterlässt charakteristische Spuren, die in weiterer Folge zur krebsfördernden DNA Mutationen führen können. In den meisten Fällen ist die zelleigene DNA Reparaturmaschinerie jedoch in der Lage diese Punktmutationen zu beheben, was den Fortbestand der betroffenen Zelle ermöglicht. Im Falle eines irreparablen Genoms wird der Keratinozyt die sogenannte Differenzierungskaskade, eine spezielle Form des kontrollierten Zelltods, einleiten und sich somit unschädlich machen. Während dieses Prozesses wandern die Keratinozyten kontinuierlich Richtung Hautoberfläche, verändern dabei ihre Erscheinung, beenden ihre metabolische Aktivität und sterben. Während dieses Prozesses und sogar nach dem Zelltod erfüllen differenzierende Keratinozyten wichtige funktionelle Aufgaben wie zum Beispiel eine physikalische Abschirmung gegen Umwelteinflüsse und Bakterienangriffe gegen den Organismus.

Falls es einem entarteten Keratinozyten gelingt, sowohl der DNA Reparatur als auch der vorzeitigen Differenzierung zu entkommen, stellt das Immunsystem die letzte Hoffnung dar, in dem es die gefährliche Zelle erkennt und neutralisiert. Aus diesem Grund haben Transplantatempfänger (OTR), unter Immunsuppression, ein erheblich erhöhtes Risiko, auch schwere Formen von SCC zu entwickeln. Normalerweise werden SCC operativ entfernt, was in den meisten Fällen eine kurative Behandlung darstellt. Ein kleiner Anteil invasiver SCC findet den Weg zu Blut- oder Lymphgefässen und breitet sich als metastasierendes SCC in weitere Organe des menschlichen Körpers aus. Die Überlebenschancen bei dieser besonders aggressiven Form des SCC sind sehr gering und die Behandlungsmöglichkeiten sehr beschränkt. Detaillierte Kenntnis über die molekularen Vorgänge während dieser invasiven Prozesse sind unerlässlich, um der betroffenen Patientengruppe in Zukunft bessere Therapien in Aussicht stellen zu können.

Physiologische und pathologische Prozesse werden nicht nur durch unterschiedliche Expression von proteinkodierenden Genen reguliert, sondern auch durch alternierende microRNA (miRNA) Spiegel. miRNAs sind kleine, nichtkodierende, ungefähr 22 Nukleotide lange RNA Fragmente, die mit der 3'UTR von mRNAs interagieren und somit deren Stabilität/Funktionalität negativ beeinflussen. Aufgrund unterschiedlicher miRNA Expressionsmuster mit den einhergehenden Funktionen im Krebs- und normalen Gewebe, erscheint eine Einteilung in krebsfördernde (sogenannte Oncomirs) und tumorsupprimierende miRNAs sinnvoll. miRNAs sind massgeblich an sämtlichen funktionalen Vorgängen in Krebserkrankungen, wie zum Beispiel Proliferation, Apoptose, Bildung von Metastasen sowie der Angiogenese, beteiligt.

Basierend auf unserer Erkenntnis, dass microRNA-181a (miR-181a) eine deutlich reduzierte Expression im SCC im Vergleich zur normalen Haut aufweist, habe ich mich in dieser Doktorarbeit hauptsächlich mit deren tumorsupprimierenden Rolle bei SCC beschäftigt. Dabei provozierte das Fehlen von miR-181a, mittels «knock-down», krebsähnliche Vorgänge wie erhöhte Proliferation und gestörte Differenzierung in normaler Haut. Umgekehrt hatte die Wiederherstellung der miR-181a Expression in SCC eine Verlangsamung der Zellproliferation sowie die Einleitung der Apoptose zur Folge. Unseren Erkenntnissen zufolge ist das Proto-Onkogen *KRAS* ein direktes Ziel von miR-181a und befindet sich unter deren Kontrolle in Keratinozyten. In unseren Experimenten konnten wir das onkogene Potential von *KRAS* in SCC verdeutlichen, welches durch den MAPK Signalweg vermittelt wird.

miR-181a wiederum wird durch bisweilen unbekannte Prozesse während der Keratinozyten-Differenzierungskaskade erhöht exprimiert und reguliert. Daher schlussfolgerten wir, dass die durch gestörte Differenzierungsprozesse verminderte miR-181a Expression zu einer Enthemmung von *KRAS* führt, welches wiederum die Tumorentwicklung begünstigt.

Ein weiteres wichtiges Tumorsuppressorgen, welches ebenfalls während der Keratinozyten Differenzierung reguliert wird, ist *CYFIP1*. Als negativer Regulator des Arp2/3 Komplexes unterdrückt *CYFIP1* die zielgerichtete Synthese von Aktinfilamenten, welche die Grundlage der Zellmigration darstellt. In unseren Experimenten konnten wir nachweisen, dass *CYFIP1* in Folge von *NOTCH1* Aktivierung ansteigt, mit dem Grad der Keratinozytendifferenzierung korreliert und ein vergleichsweise geringes Vorkommen im invasiven SCC aufweist. Passenderweise verlieren SCC Zellen mit zuvor dysfunktionalem *NOTCH1* ihre invasive Neigung, sobald *NOTCH1* künstlich wiederhergestellt wird. Ein gleichzeitiger *CYFIP1* knock-

down hob diesen Effekt wieder auf. Des weiteren konnten wir eine direkte Interaktion zwischen *CLS*, eines zum *NOTCH1* Signalweg gehörendem Transkriptionsfaktors, mit der Promoterregion von *CYFIP1* mittels ChIP-Assay nachweisen.

Die in dieser Doktorarbeit erlangten Erkenntnisse tragen zu einem besseren Verständnis der Vorgänge bei, mit Hilfe derer miR-181a und *CYFIP1* die wichtigsten krebsbezogenen Aspekte wie unkontrollierte Proliferation und erhöhte Invasion negativ regulieren. Des weiteren könnte das neue Wissen direkt bei der Entwicklung von zukünftigen, auf miRNAs basierenden SCC Therapien eingesetzt werden. Dank der Entschlüsselung der Regulationsmechanismen, welchen die SCC Invasion unterliegt, erscheint der Arp2/3 Komplex als logisches therapeutisches Ziel zur Bekämpfung von invasivem und vielleicht auch metastasierendem SCC.

2. Summary

Cutaneous squamous cell carcinoma (SCC) represents the second most common skin malignancy among the Caucasian population. Within the last decades its incidence hast been constantly rising, being, due to its given metastatic potential, responsible for up to 25% of skin cancer-related deaths worldwide. A major risk factor leading to SCC is chronic low-level exposure to solar UV light, causing characteristic DNA damage which in turn may lead to critical cancer driving mutations. The human DNA repair machinery is highly capable in fixing UV-induced mutations allowing affected keratinocytes to survive. Severe damage of keratinocyte genomes or failure of the DNA repair machinery typically leads to precocious keratinocyte differentiation, thereby eliminating these pre-cancerous cells. During this process cells migrate towards upper epidermal layers accompanied by characteristic morphologic changes and gradual metabolic shut down followed by cell death. During this cascade and even after cell death, keratinocytes fulfill important tasks such as a barrier function against harmful environmental conditions or bacterial intrusion.

When worst comes to worst and a damaged keratinocyte evades not only DNA repair, but also differentiation, the human immune system usually represents the last resort by attacking and eliminating affected cells. This circumstance explains why immunosuppressed patients bear a substantially increased risk of developing SCC. Typically, SCC are surgically excised, which represents a curative treatment. A small fraction of SCC seeds to dermal blood or lymph vessels and may eventually form distant metastasis. Metastasizing SCC is a highly aggressive form of cancer with poor survival rate, where only a few treatment options with limited impact are available. Therefore, it is substantial to gain knowledge of mechanisms and strategies used by keratinocytes to become successfully invasive tumors.

Physiologic and pathologic processes are not only regulated by changes in the expression of protein-encoding genes, but also by alterations in the levels of microRNAs (miRNAs). miRNAs are approximately 22 nucleotide-long non-coding RNA molecules binding to the 3' untranslated regions (UTR) of target mRNAs in a sequence-specific manner thus influencing translation and/or stability of the transcripts. miRNA expression analyses suggest important oncogenic as well as tumor-suppressive roles of miRNAs, referred to as tumor-driving (so called oncomirs) and tumor-suppressor miRNAs, respectively. They effectively play roles in

almost all aspects of cancer biology such as in proliferation, apoptosis, metastasis and angiogenesis.

Based on our observation that microRNA-181a (miR-181a) shows lower abundance in SCC compared to normal skin, the main aim of this PhD thesis was to investigate its role as a tumor-suppressive miRNA in SCC. Indeed, by knocking down miR-181a in normal skin, cells acquired cancerous characteristics, such as increased proliferation and disturbed differentiation. Conversely, reestablishing miR-181a in SCC cells tamed their cancerous behavior, as shown by decreased proliferation and induction of apoptosis. We confirmed that the proto-oncogene KRAS not only acted as a direct target of miR-181a, but also effectively regulated by miR-181a in keratinocytes. In our experiments, *KRAS'* oncogenic potential in SCC became evident and could be directly ascribed to the negative regulation through miR-181a. Furthermore, *KRAS* mediated its pro-proliferative stimulus via the MAPK signaling pathway.

Looking upstream, miR-181a seemed to be upregulated by processes during keratinocyte differentiation. We hence conclude that disturbed differentiation leads to reduced miR-181a levels in SCC and subsequently to critically decreased suppression of *KRAS* which in turn promotes carcinogenesis.

Another tumor suppressor gene tightly connected to keratinocyte differentiation is *CYFIP1*. As a negative regulator of the Arp2/3 complex it interferes with actin plasticity-driven cell migration and is therefore associated with the metastatic potential of SCC. Our experiments demonstrated that *CYFIP1* was strongly induced upon Notch1 activation, correlated with the grade of keratinocyte differentiation and showed low abundance in invasive SCC. Functionally, SCC cells with defective Notch1 signaling lost their invasive potential upon artificial *NOTCH1* reactivation. A simultaneous *CYFIP1* knockdown abolished this effect, driving the cells towards cancerous invasion once again. Finally, we proved the direct interaction between *CLS*, the DNA binding transcription factor of Notch1 signaling, and *CYFIP1* promotor region.

In summary, the results of the current thesis provide a better understanding of how the oncogenes miR-181a and *CYFIP1*, both activated during keratinocytes differentiation, control the main hallmarks of SCC, elevated proliferation and invasion. These novel findings may be directly applied in the development of topical miRNA treatments against SCC in the future.

Insight into SCC's regulatory network of invasion suggests the Apr2/3 protein complex as a novel therapeutic target against invasive and possible also against metastasizing SCC.

3. Introduction

4.1 Skin

The skin is the largest human organ accounting for 1.6 – 2 m^2 depending on the body size. The main task of the skin is protection of the organism against environmental conditions such as pathogens, toxins, physical stress and climate factors [1]. The organ structure can be divided into the outer epidermis, the much thicker dermis and the subcutaneous hypodermis consisting of subcutaneous fatty tissue [2]. The dermis is mainly composed out of collagen giving it a coriaceous structure. Fibroblasts, mast cells, histiocytes and a large proportion of extracellular matrix form the substance crisscrossed by blood capillaries. Dermis and epidermis are separated by a thin membrane composed out of collagen IV and laminin called basal membrane on which the epidermal cells are anchored. Epidermis and dermis are separated by the basal membrane (**Figure 1**). The epidermis accounts for 0.05 mm to 1.5 mm, while the thickness of the dermis ranges between 1.5 mm and 4.0 mm, depending on the body site [3]. Keratinocytes are the predominant cell type found within the epidermis, accounting for around 95% of the cells. According to their differentiation status the epidermis can be vertically divided into stratum basale, stratum spinosum, stratum granulosum and the outer most stratum corneum [4].

Hair follicles are dispersed over large parts of the organ, housing stem cells for keratinocytes and other cell types. A variety of glands ensure skin integrity, water tightness and temperature control by excreting different substances [5].

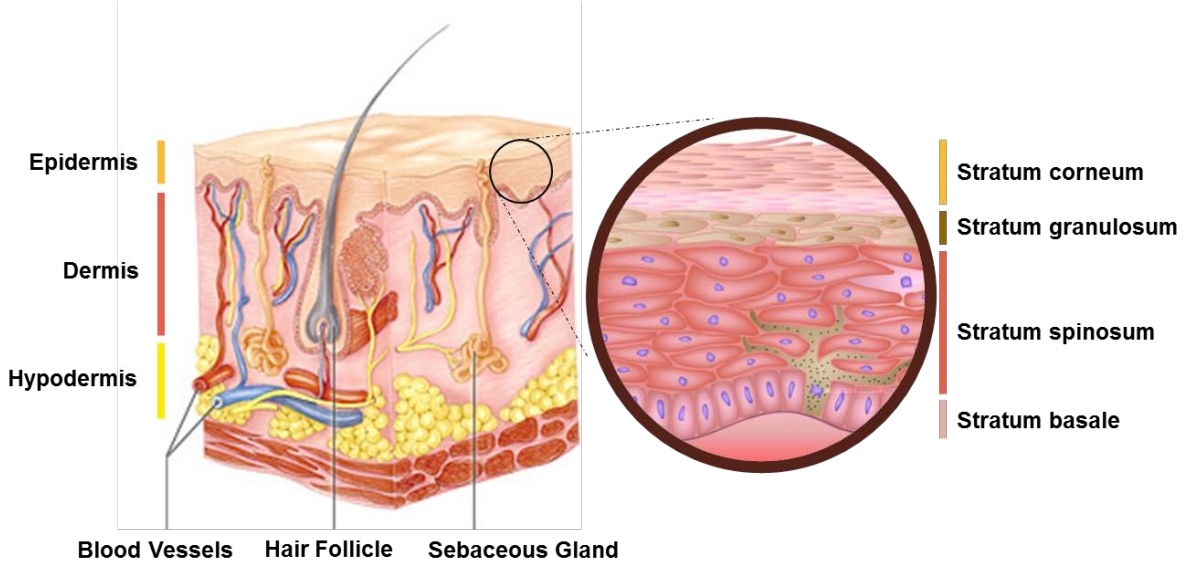

Figure 1: Structure of the human skin

The figure is based on illustrations from webMD.com and skinpilot.de.

4.1.1 Cells of the skin

4.1.1.1 Keratinocytes

The keratinocyte stem cell pool can be found in hair follicles. In their relatively short life span of approximately 28 days they spread across the basal lamina and travel towards superficial epidermal layers while differentiating [6]. While basal keratinocytes are proliferative, cells of outer layers gradually shut down their metabolism and disperse their nuclei resulting in cell death. As an important side effect, differentiation of damaged keratinocytes effectively neutralizes their oncogenic potential. Furthermore, even terminally differentiated keratinocytes are still fulfilling important tasks as “functional corpses” [7]. While their differentiation status can be determined relatively easy using microscopy, their molecular classification relies on the correct interpretation of differentiation markers. Certain keratins like keratin 5 and 14 are mainly found in the proliferating cell layer. Keratin 1 and 10 are characteristic for the spinous and granular layer. Especially proteins of the cornified envelope like involucrin, filaggrin and loricrin are markers for more advanced differentiation found in the stratum corneum [8, 9]. Flaws in the differentiation cascade may lead to keratinocyte cancers. Cutaneous squamous cell carcinoma (SCC), for example, is, among other criteria, characterized by a disturbed differentiation pattern. Histologically conspicuous features are

abnormal thickening of differentiated cell layers, called hyperkeratosis, often accompanied by the presence of nuclei, referred to as parakeratosis [10].

4.1.1.2 Melanocytes

Unlike other skin cells melanocytes originate from the neural crest and travel towards their destination, the epidermal basal lamina, during embryonic development [11]. Their main task is production and distribution of melanin among keratinocytes, protecting their DNA from UV-induced damage [12]. Benign accumulations of melanocytes form moles or “nevi”, while a malignant transformation to melanoma causes most skin cancer relates deaths (American Cancer Society, 2016-08-11).

4.1.1.3 Merkel cells

Merkel cells are associated with nerve endings within the basal epidermal layer and function as mechanosensory organs. Malignant transformations of these cells are very rare, but associated with high mortality [13].

4.1.1.4 Fibroblasts

Fibroblasts are the predominant dermal cells and produce the fibrous structure giving the skin its robustness. In addition, they play important roles in wound healing and communicate closely with epidermal keratinocytes [14, 15]. Defects in this communication network may lead to formation of SCC on sun damaged skin [16].

4.1.1.5 Immune cells

To effectively protect the body against pathogens, the skin is not only a physical barrier but an active immune organ [17]. A variety of immunocompetent cells with migratory capacity, such as leukocytes, mast cells, monocytes and macrophages can be found in the dermis and hypodermis. As mentioned before, the dermis is equipped with lymph and blood vessels and contains the majority of immune cells, including, T cells, dendritic cells and others. Therefore, most of the immune processes are taking place there. The epidermis, lacking direct access to blood or lymph vessels, is mainly equipped with antigen-presenting Langerhans cells, dendritic epidermal T cells and relies on basic immune functions of keratinocytes and melanocytes [18].

4.2 Cancer

4.2.1 Basics of cancer – hallmarks of cancer

In complex multi-cellular organisms, such as mammals, every aspect of cellular behavior needs to be regulated precisely to ensure flawless operation of the collective.

Tumor cells vividly demonstrate the sensitive balance of cellular regulatory mechanisms. A slew of critical events randomly occurs, thus the control over basic functions of the cell is lost. Once a cell evades control and immune system clearance, it gains a strong selection advantage over normally functioning cells. The consequences are excessive proliferation followed by gradual spreading over large parts of the body. Cells of various tissues will be affected, disturbing adequate organ function and eventually leading to death [19].

In two reports Hanahan and Weinberg summarize the generally accepted cancer characteristics as “Hallmarks of Cancer” [20] (**Figure 2**). The first report discusses how proliferative signaling, evading growth suppressors, onset of metastasis, replicative immortality, induction of angiogenesis and resistance against cell death enable cancerous cells to survive, grow and spread. In their second issue “Hallmarks of Cancer: The next generation”, they broaden their discussion, taking so called “enabling” criteria, such as chronic inflammation, adaptations and increased flexibility acquired through genomic instability into account [21]. Recent pharmaceutical approaches successfully target these hallmarks in some malignancies e.g. by kinase inhibition, whereas the cancer in turn quickly reacts by acquiring resistance mechanisms [20], making continued research and development indispensable.

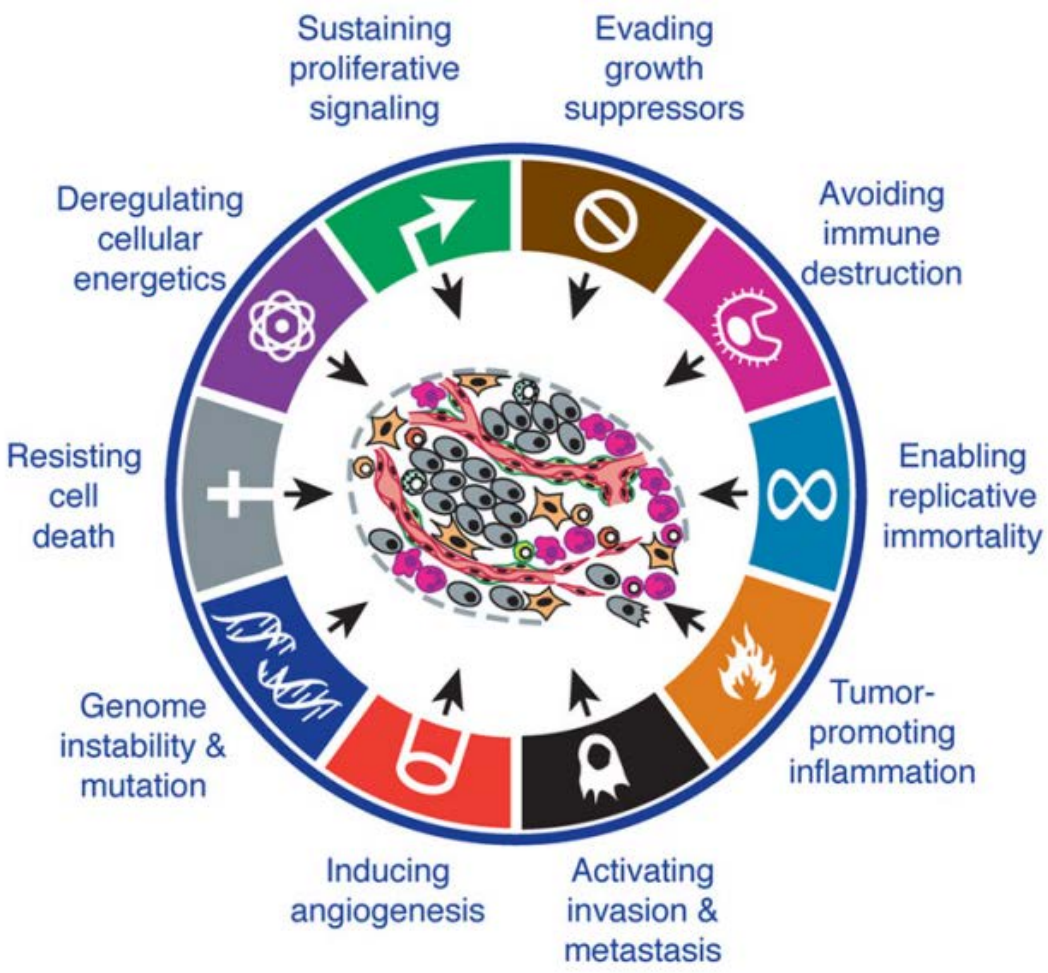

Figure 2: Hallmarks of Cancer: The next generation [21]

4.2.2 Skin cancer

4.2.2.1 Melanoma and non-melanoma skin cancer

Skin cancer is roughly divided into melanoma and non-melanoma skin cancer depending on the affected cell type. Melanocytes, pigmented cells found in the basal epidermal layer, give rise to melanoma [11]. Based on statistical investigations in the US, the National Cancer Institute of the NIH estimates 76,380 new cases of melanoma representing 4.5% of all new cancer cases in 2016. The disease is responsible for 10,130 deaths in the US per year, corresponding to 1.7% of all cancer related deaths [3]. Since the early 1990s melanoma incidence has been constantly rising while the number of resulting deaths remains relatively constant. This improved outcome is mostly due to early recognition of thin tumors and also to advances in treatment options, such as a variety of small molecule inhibitors or highly effective immune therapies. Furthermore, the effort invested into increasing the population's

awareness led to earlier diagnosis of Melanoma. In its early stage, where no vessel invasion and metastasis formation takes place, the 5-year survival rate is 98.4% and drops dramatically to 17.9% at later stages when distant metastasis are present [3].

All other skin cancers originate from cells other than melanocytes, and are referred to as non-melanoma skin cancer [22]. The great majority, approximately 80%, of all non-melanoma skin cancers are basal cell carcinomas (BCC). Although the incidence has been constantly rising to three million Americans diagnosed in 2016 [19], BCC-related deaths are rare, mostly due to its low metastasizing potential. Furthermore, the available treatment options, for the majority surgical excision and rarely hedgehog pathway inhibitors result in reliable BCC clearance or control [23-25]. Previously, BCC was thought to arise from the basal keratinocytes, while later studies consider keratinocyte stem cells of the hair follicle bulge as its origin [26, 27].

The second-most frequent non-melanoma skin cancer is SCC which typically arises from keratinocytes found on the basal epidermal layer or from hair follicle-associated cells [28, 29]. Other non-melanoma skin cancers are Merkel cell carcinoma, cutaneous T-cell lymphoma or Kaposi's sarcoma, for example, accounting for less than 10% of skin cancers, depending on the geographical region and other factors [30].

4.2.2.2 AK as in-situ SCC and invasive SCC

4.2.2.2.1 Overview

In-situ SCC in the form of actinic keratosis (AK) or Bowen's disease is typically found on sun exposed sites of the body, such as the face, lower arms and the balding head [31]. Depending on the vertical spreading within the epidermis, AK can be classified as keratinocyte intraepidermal neoplasia (KIN) I – III. KIN I describes AK of the basal third of the epidermis, KIN II affects the lower two thirds and KIN III concerns the whole epidermis [32]. AK is histologically characterized, among other features, by abnormal keratotic thickening and the presence of nuclei in terminally differentiated keratinocytes, referred to as hyperkeratosis and parakeratosis respectively. Since chronic UV exposure is seen as the major AK cause, these histologic hallmarks are found in a pattern intermitted by hair follicles, which provide light protection resulting in unaffected epidermal regions within their proximity. As AK progresses to invasive SCC, it acquires more general cancer characteristics, such as pleomorphic cells, hyperchromatin, hypereosinophilia or immune cell infiltration. More SCC-specific structures

are small nests of keratinization within the tumor, such as keratin pearls, consisting of more differentiated spots. Furthermore, various cyst-like structures of terminally differentiated keratinocytes can be found, making SCC histologically a highly irregular tumor [33, 34] (**Figure 3**). Since poorly differentiated SCC is associated with worse patient outcomes, the overall differentiation state may offer valuable clues towards cancer prognosis [35].

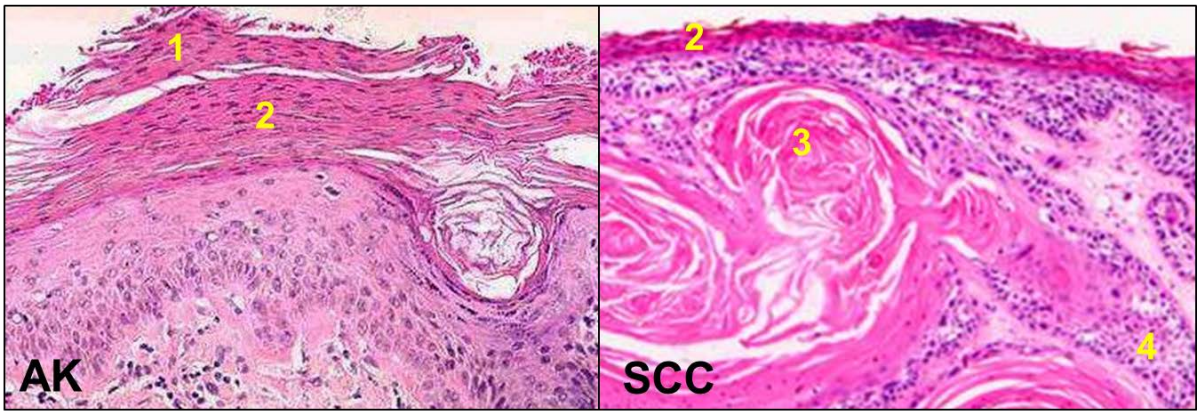

Figure 3: Histological features of AK and SCC.
(1) hyper keratosis (2) parakeratosis (3) keratotic pearl (4) immune cell infiltration.
Modified from [34] and http://www.mrcophth.com.

SCC is typically found in elderly Caucasians with fair skin, where males are significantly more likely to be affected than females. SCC incidence has dramatically increased within the last decades with more than one million cases diagnosed in the U.S. each year and a death rate of approximately 2% [36, 37]. Although the population's awareness of the risks posed by solar UV is steadily increasing, the long latency of the disease is made responsible for the current situation where older generations are mainly involved. Geographically, regions with high UV exposure, like Australia and New Zeeland, are most affected [38]. Chronic low-level exposure to UV, during leisure time and up in the mountains for example, have a major impact on SCC formation, possibly explaining the high incidence in countries like Switzerland.

4.2.2.2.2 Molecular pathogenesis of SCC

In order to fulfill their functions in the human body, keratinocytes need to be permanently subject to tight control communicated via complex cell signaling pathways. The most important carcinogenic factors and the according molecular consequences which may lead to SCC formation will be introduced in this section.

4.2.2.2.3 The impact of UV light

Typical sites for SCC formation are the face, balding head, lower arms and the dorsum of the hands, indicating the crucial role of chronic ultra violet (UV) exposure in tumor development and progression. According to their wave length and the correlating energy, UV light is separated into UVA (320 nm – 400 nm, low energy), UVB (280 nm – 320 nm, medium energy) and UVC (< 280 nm, high energy). Due to atmospheric absorption the natural occurrence of UVC is rare and has an accordingly minor role in SCC development [39]. In contrast, solar UVA and UVB light penetrate deep into epidermal and dermal skin layers with severe impact on the cells [40]. UVB directly induces chemical reactions leading to DNA mutations during DNA replication. Neighboring pyrimidines are most vulnerable as UVB irradiation frequently leads to formation of cyclobutane pyrimidine dimers (CPD)or pyrimidine (6-4)pyrimidones (64PPs). The pyrimidine cysteine within these photoproducts is unstable and tends to deaminize spontaneously, leaving an uracil instead. Since uracil is under natural circumstances found exclusively in the RNA, but not in the DNA, it is replaced with the according DNA base thymidine during DNA repair resulting in C -> T mutations [41, 42]. To some extent UVA light is known to induce these direct photo reactions as well, but the more common UVA reaction is the generation of reactive oxygen species (ROS). ROS attack DNA causing double-strand breaks and derivatization of guanine to 8-hydroxyguanine (8OH-G), which in turn may result in point mutations [43].

The importance of permanent and reliable DNA repair is vividly demonstrated by patients suffering from xeroderma pigmentosum (XP), a rare autosomal recessive genetic disorder of DNA repair. Any UV-induced DNA damage here is translated largely unbridled into cancer formation [44, 45].

4.2.2.2.4 The impact of immunosuppression

Organ transplant recipients (OTR) carry a dramatically elevated risk of forming SCC [46]. More specifically, this patient group faces a life time risk of up to 90% to suffer from SCC and a 250-fold increased risk relative to the general population. Furthermore, they are exposed to an increased risk of forming severe forms of SCC, such as field cancerization or metastasizing SCC [47]. In turn, SCC represents by far the most common cancer within this patient group [48]. The restrained immune system clears cancerous cells within the skin less effectively resulting in improved survival of malignant cells in turn leading to full-blown cancer. Kidney and heart transplant recipients receive higher doses of immunosuppressants compared to liver transplant recipients, where lower doses are sufficient to prevent organ rejection. SCC occurrence among those patient groups clearly correlates with the degree of immune suppression further underlining the importance of a competent immune system in maintaining skin homeostasis [49, 50]. Besides these indirect effects, many studies point out various direct mechanisms in which the immune suppressing drugs itself, such as the calcineurin inhibitor cyclosporine A (CsA), promotes cancer formation [51]. Research in our own laboratory, contributed to the understanding of how the AP1 transcription factor family member, activating transcription factor 3 (ATF3) is potentiated through two different signaling pathways to induce excessive keratinocyte proliferation. On the one hand UVA induced *ATF3* expression through ROS-mediated nuclear factor erythroid 2-related factor 2 (NRF2) activation while nuclear factor of activated T cells (NFAT), a negative regulator of *ATF3*, was suppressed by CsA mediated calcineurin inhibition. Induced *ATF3*, subsequently, inhibited p53 expression resulting in keratinocyte carcinogenesis (**Figure 4**) [52].

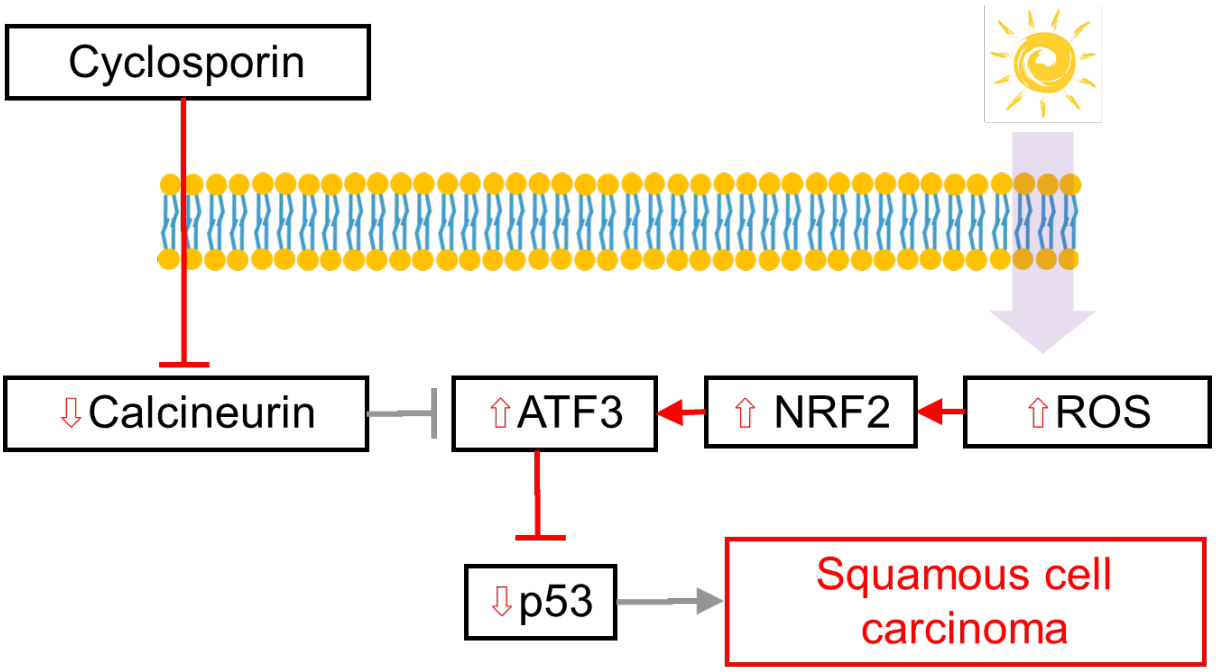

Figure 4: CsA in combination with UV light potentiates ATF3 to form SCC in OTR patients [52]

Another immunosuppressive drug, azathioprine, has been shown to directly generate a chaotic pattern of DNA mutations in combination with UV light. 6-thioguanine, the active compound of azathioprine, functions as a DNA/RNA analogue and is incorporated into the genome of, mainly immune cells with consequences to their function. As a side effect, 6-thioguanine generates ROS upon UV absorption which in turn may lead to critical cancer causing mutations in keratinocytes [53].

Mammalian Target of Rapamycin (mTor) inhibition represents an alternative strategy for OTR to achieve adequate immunosuppression, causing fewer side effects and a much lower DNA mutation frequency. After the introduction of mycophenolate mofetil, which benefits from antiproliferative properties combined with unchanged photosensitivity in patients, azathioprine was gradually pushed back from the market [54].

4.2.2.2.5 Characteristic mutations in SCC

Primary melanoma, for example, is characterized by very frequently occurring mutations in *BRAF* and *NRAS*. The situation in SCC, on the other hand, is much more heterogeneous with a diffuse pattern of mutations, largely unrelated to SCC staging. However, there are a few mutations prevalent in SCC, which will be summarized in the following paragraphs [55].

4.2.2.2.5.1 P53

P53, or tumor protein *p53* (*TP53*) in humans, plays a central role in preventing UV induced carcinogenesis and is nicknamed “the guardian of the genome”. In its steady state *p53* is subject to a balance of constant production. DNA damage leads to chemical modification of *p53*, reducing its degradation and thereby resulting in p53 accumulation [56]. Stabilized *p53* activates multiple tumor suppressive pathways like *p21* activation and initiation of DNA repair mechanisms leading to cell cycle arrest [57] degradation [58, 59]. In case of failed DNA damage repair the cell is usually forced to undergo apoptosis or differentiation, in the case of keratinocytes [60]. Mutated *p53* loses its DNA binding capability and fails to activate the sequential tumor suppressive gene expression cascade [61]. A large proportion of non-melanoma skin cancers harbor *p53* loss-of-function mutations in association with a clear UV signature on their DNA. 60% of AK are associated with *p53* mutations, and a study on invasive SCC, for example, observed *p53* mutations in 58% of the cases [62, 63]. Notably, *p53* mutations can already be found in normally appearing keratinocytes of UV exposed skin areas.

4.2.2.2.5.2 Notch signaling

Notch signaling represents a very basic pathway for cell-cell communication, is highly conserved and therefore found in almost all multi-cellular organisms [64]. Notch ligands are commonly cell surface components and require direct cell-cell contact in order to activate Notch signaling. Thereby, groups of neighboring cells gain the ability to organize themselves as observed in lateral or contact growth inhibition. On top of that, cell-cell contact signaling via Notch can determine cell fate and plays crucial roles during embryonic development. Mammals possess four different Notch receptors, referred to as *NOTCH1*, *NOTCH2*, *NOTCH3*, and *NOTCH4* [65, 66].

As a single-pass membrane protein, Notch receptor spans the cell membrane and can be divided into an extra- and intracellular domain. Upon ligand binding to the extracellular domain, the signal is transferred to the intracellular domain, where proteolytic cleavage takes places [64, 67]. This reaction releases a component of the intracellular domain which translocates into the nucleus where it associates with the DNA-binding protein *CSL* and an ancillary protein, MamL1 or related family members. The resulting transcription factor complex interferes with target gene expression. Among others the best characterized targets of *NOTCH1*, the prevalent family member in the skin, are *HES1*, *p21* and *IRF6* [68, 69].

While Notch activation in the skin is mainly connected to cellular differentiation and suppression of growth, its depletion in the brain leads to precocious neural differentiation during development. Therefore, Notch acts as a tumor suppressor as well as an oncogene depending on the organ, making the signaling pathway highly context specific [70, 71].

Human skin relies on functioning Notch signaling to maintain its homeostasis. Keratinocyte differentiation is triggered by Notch activation, and loss of function may cause skin cancer formation [72]. The molecular mechanisms downstream of Notch activation that elicit differentiation remain elusive.

The communication between dermis and epidermis partly relies on Notch signaling. Hu and colleagues showed in an *in vivo* model, that mice with dermal *NOTCH1* depletion rapidly form SCC on multiple sites of the body [16]. In vitro SCC cell lines like SCC13 are unable to differentiate due to defects in *NOTCH1* signaling, which can be seen as the main factor responsible for their rapid and uncontrolled proliferation. Artificial *NOTCH1* knock in reestablishes their capability to differentiate and rescues their cancerous behavior [69].

4.2.2.2.5.3 ΔNp63 (p40)

P63, a *p53* family member, is highly expressed in squamous epithelium and to some extent also in normal basal cells, myoepithelial cells, trophoblasts, thymic epithelium and urothelium. P63 has two predominant and clinically relevant isoforms, *TAp63* and *ΔNp63* with opposing functions [73]. While *TAp63* functions as a tumor suppressor, similar as *p53*, *ΔNp63* acts as its agonist and can therefore be seen as an oncogene [74, 75]. Detectable *ΔNp63* expression is very specific to SCC allowing reliable distinction between normal skin and other neoplasia in routine clinical diagnostics [76].

4.2.2.2.5.4 Wnt signaling

Wnt signaling has recently been identified as altered in SCC with evidence for functionality in SCC formation and progression [55, 77].

In the absence of Wnt in canonical Wnt signaling, β-catenin is subjected to a fine balance of permanent expression and degradation. Upon activation through Wnt ligands binding to seven-pass transmembrane-containing Frizzled (*FZD*) receptors, β-catenin degradation is interrupted resulting in accumulation and translocation into the nucleus where oncogene expression is initiated [78]. Different canonical and non-canonical Wnt signaling variants are summarized under the term Wnt signaling network [79, 80]. LGK974, a small-molecule Wnt signaling inhibitor, is well tolerated, and highly efficacious in human head and neck SCC cells [81], suggesting that it may prove beneficial in other SCCs with hyper-activated Wnt/β-catenin signaling [77].

4.2.2.2.5.5 TERT

The *TERT* (telomerase reverse transcriptase) gene encodes for the catalytic subunit of telomerase. By elongating the chromosomal telomeres, the enzyme prevents their shortening during DNA replication allowing the cells to maintain stemness and become potentially immortal. This mechanism is used by various cancer cells to evade senescence and apoptosis during uncontrolled proliferation, making *TERT* mutation a common feature found in BCC and SCC [82].

4.2.2.2.5.6 CDKN2A

The *CDKN2A* (cyclin-dependent kinase inhibitor 2A) gene encodes for *p16*, a potent tumor suppressor protein. By binding to cyclin-dependent kinases 4 and 6 it prevents their interaction with cyclins, leading to G1 cell cycle arrest. Additionally, *p16* is known to induce Notch-dependent differentiation in keratinocytes. Therefore, cancer cells acquiring *CDKN2A* mutations evade senescence and cell-cycle arrest [83, 84].

4.2.2.2.5.7 The Ras family

Mammalian cells express, among others, three closely related Ras proteins: *HRAS*, *KRAS* and *NRAS* known as the core Ras family. Mutationally activated at codons 12, 13 or 61, these small GTPases promote oncogenesis in a variety of organs, such as the lung, pancreas or intestine

[85], mostly by overactivating MAPK signaling [86]. The molecular mechanisms and other facts concerning small GTPases, specifically Ras family members, will be described in more detail below.

4.2.2.2.5.7.1 KRAS

Kirsten rat sarcoma viral oncogene homolog (*KRAS*) is a small GTPase. This class of signaling molecules characterized by hydrolase activity can bind and hydrolyze guanosine triphosphate (GTP) to form guanosine diphosphate (GDP). In its inactive state it is bound by GDT while association with GTP activates *KRAS*.

So called GTP-exchange factors (GAP) activate *KRAS* by swapping GDP with a GTP molecule. Given that small GTPases possess hydrolase activity, they can switch themselves off by processing GTP into GDP. Due to their low enzymatic potential, however, small GTPases are supported by GTPase activating proteins (GAP) accelerating the process drastically [87, 88].

KRAS plays a central role in cell signaling. Upstream it is typically activated by receptor tyrosine kinases (RTK) and downstream KRAS activates various signaling pathways regulating various functional aspects in normal tissue (**Figure 5**). A single amino acid mutation however can prevent GTP hydrolyzation leading to constitutively active KRAS as seen in many cancers, such as colon cancer or non-small cell lung cancer (NSCLC) [89-91].

Besides mutations of *KRAS* itself, several circumstances may lead to critical gain of function. Jammed *KRAS* degradation, elevated transcription or faulty posttranscriptional control through miRNAs are to be mentioned as examples [92, 93].

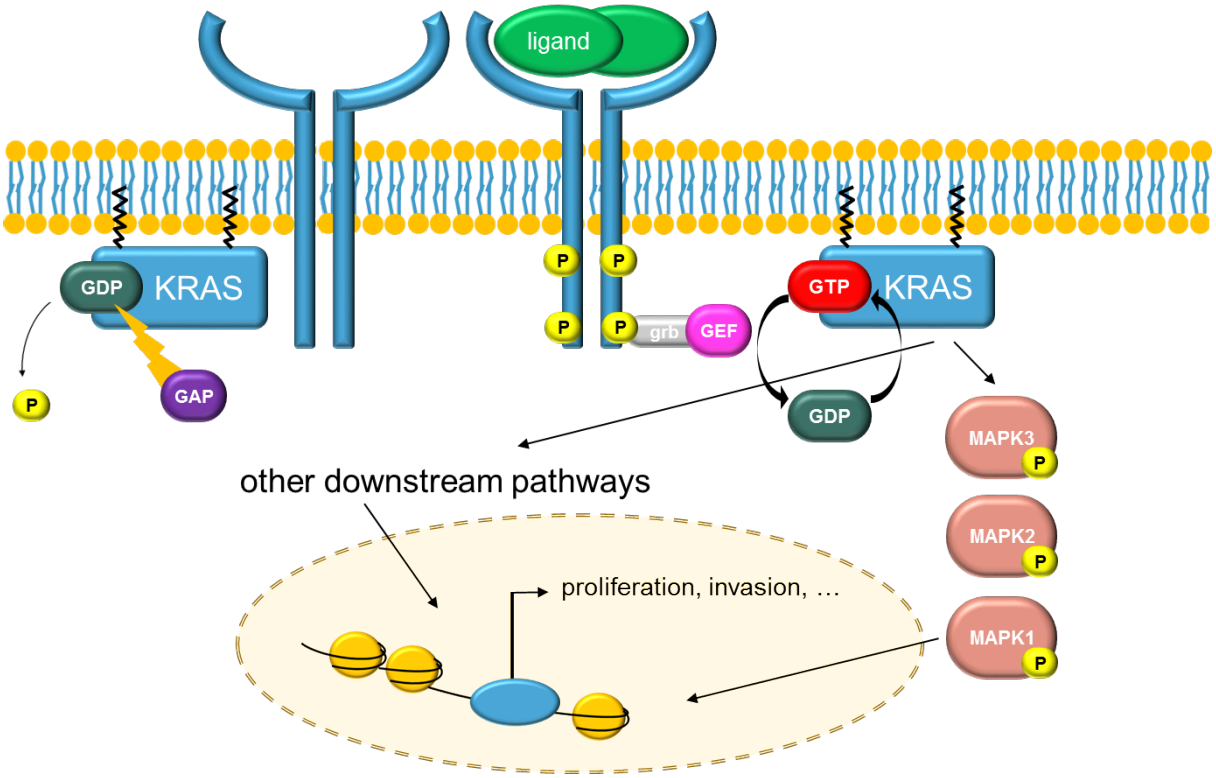

Figure 5: Illustration of small GTPases (or *KRAS*) and their major mediators.

4.2.2.2.5.8 MAPK signaling pathway

The mitogen activated protein kinase (MAPK) signaling pathway consists of a cascade of typically three kinases which are successively phosphorylated. The core MAPK branch consists of *RAF – MEK – ERK* and is usually activated by Rho or Ras family members such as *KRAS*. Other canonical branches involve *p36*, *JNK* and *ERK5*. In healthy tissue several MAPK branches regulate functional aspects from metabolism to apoptosis in concert. Thereby, Extracellular regulated *MAP* kinase 1/2 (*ERK*) usually takes over stimulatory roles while *p38* for example has a rather decelerating character (**Figure 6**) [94, 95]. Due to its central role in cell signaling the MAPK pathway is frequently hyperactive in cancer, either due to activating mutations of its own kinases, *BRAFV600E* for example, or by uncontrolled upstream activation, through growth factor receptors and GTPases [86, 96]. As discovered in our own research, MAPK signaling exhibits a controversial role in SCC. While a mild activation, achieved by suppressing negative regulators for example, is connected to carcinogenesis, hyperactivation may result in oncogene-induced senescence [97].

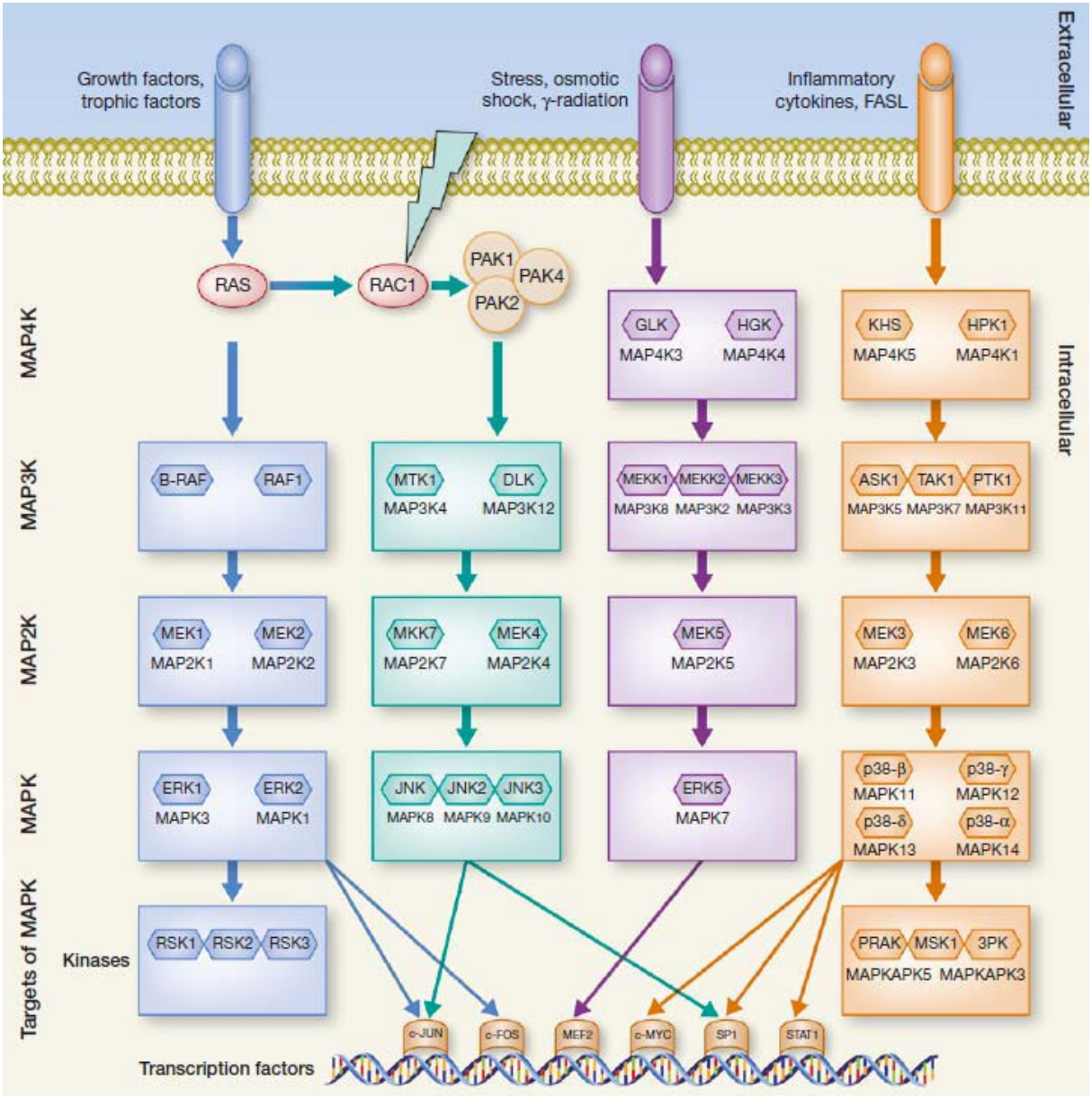

Figure 6: Branches of MAPK signaling [94].

4.2.2.2.6 Prevention of SCC

Since UV light is seen as the major cause for AK and SCC formation, awareness and prevention against its dangerous potential are advised. Long sleeve clothing and the use of sun screen provide the most effective protection against UV irradiation of the sun [98]. Individuals with immune suppression, such as OTR or HIV positive patients, are much more likely to develop SCC when exposed to UV light. Keeping track of their daily UV exposure, by the use of UV sensors, may help these patient groups to effectively reduce their risk of developing UV induced SCC [99].

4.2.2.2.7 Treatment options against AK and SCC

The earlier treatment for SCC is initiated, the fewer the complications that are to be expected and the higher the success rates. Therefore, diligent treatment of AK as in-situ SCC is advised [100]. In the context of SCC treatment, one needs to distinguish between different stages of SCC, where topical treatments in the form of creams are rather applied in early stages of AK. Field cancerization typically undergoes field treatment, while single lesions are subject to cryotherapy. Invasive SCC are excised surgically to prevent metastasis formation [101].

4.2.2.2.7.1 Photodynamic therapy

Photodynamic therapy (PDT) is a frequently applied topical treatment mainly against AK. Aminolevulinic acid as a prodrug is applied to the treatment area. After or during conversion of this prodrug to the active form of protoporphyrin IX, the photodynamic effect is induced by daylight or selective light sources [102]. As a result, ROS are generated, resulting in destruction of cell walls and secondary tumor ischemia. [103, 104]. PDT results in sustained lesion clearance in 78% to 100% of all cases, making it a very reliable and successful treatment option [105, 106].

4.2.2.2.7.2 5-Fluorouracil

5-Fluorouracil (5-FU) is a chemotherapeutic agent, aiming to interrupt thymidine synthesis and thereby DNA replication. With intermediate treatment duration and in spite of transient inflammatory reactions, it is still used nowadays [107].

4.2.2.2.7.3 Diclofenac

Diclofenac exhibits anti-inflammatory effects by inhibiting cyclooxygenase-2 (*COX-2*) and is known to induce apoptosis in cancer [108]. Diclofenac is applied topically in combination with hyaluronic acid two times a day for up to three months. Sustained lesion clearance is achieved in up to 40% of the cases [109].

4.2.2.2.7.4 Imiquimod

Imiquimod stimulates the innate immune system by binding to Toll like receptor 7 (*TLR 7*), leading to an interferon-α response mediated by NFkB signaling [110]. Its efficacy has been investigated in several studies with varying results. Cancer clearance was observed, on

average, in around 50% of the cases depending on the study. The treatment duration accounts for 16 weeks and the compound is usually well tolerated provoking relatively mild skin reactions [111, 112].

4.2.2.2.7.5 Ingenol mebutate

Ingenol mebutate (IM) was recently approved under the name Picato for the treatment of AK. It is distinguished from other topical treatments by an extremely fast response rate of only hours after application. The cream containing IM has to be applied for only two to three days, resulting in cancer clearance in more than 50% of the patients, depending on the clinical study [111, 113]. Results from our own group identified IM as a strong protein kinase C (*PKC*) agonist, which in turn overactivates the MAPK signaling pathway [97]. Unlike in many other cancers, excessive MAPK activation leads to induction of apoptosis in keratinocytes, referred to as "oncogene-induced senescence" sometimes [114]. Furthermore, IM is known to induce inflammation and necrosis resulting in temporary disfiguration. IM's simple use results in high patient compliance and can be self-applied conveniently at home.

4.2.2.2.7.6 Radiotherapy

Radiotherapy is also used to treat AK and SCC field cancerization on larger surfaces. Due to its excellent cosmetic outcome it is often applied on facial and balding scalp regions. The typical procedure comprises six sessions within a period of three weeks. Although mild X-rays cause very few side effects, the risk of secondary malignancies in the long-run should be considered [101, 115].

4.2.2.2.7.7 Surgical methods

As mentioned above, SCC holds a substantial risk of metastasis, increasing with disease progression. Therefore, invasive SCC is usually excised with a generous safety margin and subsequent histologic checkup to ensure complete tumor clearance [116]. In order to keep the safety margin as small as possible on critical body sites like the face, excision and histological checkup are executed in parallel in a step by step manner, known as Mohs surgery [117].

A quicker and therefore cheaper method is cryotherapy, where single lesions are iced by directly applying a stream of liquid nitrogen. Although this technique is highly efficient, it is limited by the hypopigmentation associated with intense use [118].

4.2.2.2.7.8 Therapies against metastatic SCC

Up to date there is no curative treatment available for metastasizing SCC. Depending on the individual situation, a combination of radiotherapy and chemotherapy may come into operation accompanied by surgical excision of accessible metastasis. Chemotherapeutics, such as cisplatin or doxorubicin, induce severe side effects, yield uncertain success and have therefore a rather palliative character [119, 120]. Due to good results achieved by immune checkpoint blockade and targeted therapies, using small molecule inhibitors, in melanoma, some studies are currently investigating their therapeutic potential against metastatic SCC [121].

4.2.2.2.7.9 Future miRNA treatment

Reagents, derived from transfection agents and applied like a cream, are currently being optimized for efficient RNA/DNA delivery into profound layers of the human skin. These vehicles could give rise to a microRNA based treatment against SCC in the future [122, 123]. This topic is discussed in the microRNA part in more detail.

4.2.2.3 Metastatic SCC

While BCC rarely metastasizes (0.0028 – 0.05% of all cases), SCC holds a substantial risk of forming near or distant metastasis (0.1 – 10% of all cases) associated with poor patient outcome and only 25 - 50% 5-year survival rate [124]. Common risk criteria for SCC to form metastasis are tumor size greater than 2 cm, advanced depth of invasion and critical sites of lesions [125, 126]. Presumably, a thick subcutaneous fat layer provides some protection against invading SCC cells, explaining the increased metastatic probability reported form SCC of the ear, where no significant fat layer is found [101]. Other risk factors are poor differentiation, and conspicuous histological subtypes, such as desmoplastic SCC [127].

A generally accepted circumstance promoting metastasis formation in SCC is immunosuppression especially via calcineurin inhibition. Hojo and colleagues demonstrated the direct effect of cyclosporine on cell morphology and behavior. Prominent pseudopods, increased cell motility, and invasive growth, which may be related to transforming growth factor β (*TGFβ*) production, could be ascribed to cyclosporine exposure [128].

4.2.2.3.1 Cellular invasion

In order to leave the primary tumor site and spread into distant regions of the human body, cancer cells must undergo a number of molecular and morphologic changes. Epithelial to mesenchymal transition (EMT) is a process used by many cancer cell types to leave their habitat and invade blood or lymph vessels. Cell adhesions, like desmosomes and hemidesmosomes, are cut and matrix degrading proteases are activated enabling the epithelial cell to adopt more mesenchyme-like features and move within tissues [129-131]. Another EMT feature is loss of basal-apical polarity and cytoskeletal rearrangements resulting in active migratory movements as described in more detail in the following section.

4.2.2.3.2 Cellular motility

A variety of cells in the human body, like immune cells or various stem cells, exhibit migratory capabilities [132, 133]. While sperm cells, for example, use their flagellum to propel themselves through liquids, most of the other motile cell types adopt amoeboid strategies allowing them to glide through the relatively tight structures of organ tissues [134, 135]. This

movement is achieved by extension of the cell membrane, alternating detachment and attachment to the substratum, forward flow of cytosol and retraction of the rear cell compartment [136, 137]. Formation of distinct membranous protrusions, so called lamellipodia, filopodia and cell membrane ruffles, can be observed on the leading edge while the main body mass is located at the trailing edge. The movement is mediated by a combination of reorganizations comprising the cell membrane and the cytoskeleton. Rear membrane sections are endocytosed at the trailing edge, transported into the direction of movement and reinserted into leading edge membrane sites, resulting in a membrane flow and gliding-like cellular movement [138, 139], referred to as "blebbing or membrane flow" [140, 141]. The driving force is mainly mediated by dynamic and site specific polymerization and demolition of actin fibers, resulting in a treadmill like motion and bulging of lamellipodia [137, 142]. *De-novo* synthesis of actin filaments is catalyzed by the Actin related protein2/3 complex (Arp2/3) in the leading edge, underlying a complex molecular regulation. Rho GTPase family members (e.g. RhoA, Cdc42 and Rac) function as molecular switches, processing signaling inputs into directed actin assembly by Arp2/3 complex. This intersection is relayed and enhanced by a family of regulatory proteins, including Wiskott–Aldrich syndrome protein (*WASP*), neural (*N*)-*WASP* and *WASP* family verprolin-homologous (*WAVE*) proteins [143-145]. This complex group of regulatory proteins is sometimes referred to as the *WAVE* complex which can be seen as an activator of Arp2/3 propelling cellular migration. Despite its activation role the *WAVE* complex harbors some negatively regulating subunits that may act as tumor suppressors (**Figure 7**). A very important candidate in this context is Cytoplasmic *FMR1* interacting protein 1 (*CYFIP1*) which is disabled in many epithelial cancers and its loss is connected with worse outcomes [146] [147]. Histological data collected by the publicly available database "Human protein Atlas" (www.proteinatlas.org, 2016-10-11) shows weak to moderate cytoplasmic *CYFIP1* abundance in the majority of invasive cancer tissues suggesting tumor-suppressive significance far beyond epithelial cancers. *CYFIP1* is a *RAC*-1-interacting protein which transmits signals from *RAC1* to the Arp2/3 complex by modulating the activity of the WASP family members, *WAVE1-3*, within the WAVE complex [148]. Furthermore, in vitro models show downregulated *CYFIP1* during cellular invasion and migration. *CYFIP1*-mediated depletion of *WAVE* function reduced epithelial adhesion and led to disorganization of tissue architecture [147].

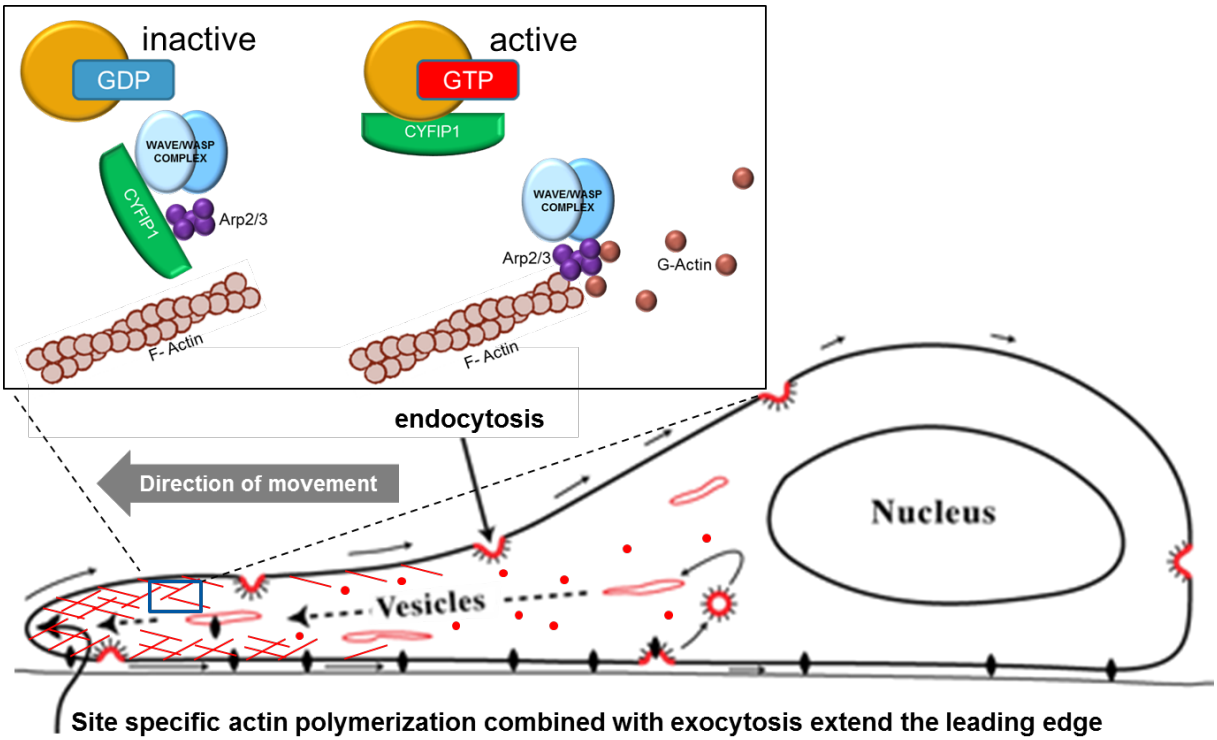

Figure 7: Mechanisms of keratinocyte cell migration.

Modified from http://jonlieffmd.com

3.3 Strategies of gene regulation / differential gene expression

Every cell of the human body possesses practically the same genetic information and still undergoes individual cell type specific differentiations enabling it to fulfill its highly specified duties. The dogmatic succession alone, describing DNA transcribed into mRNA followed by protein translation [149], cannot explain the versatile outcomes of cellular development and homeostasis. A variety of control mechanisms is necessary enabling different interpretations of the same genetic information underlying each cell. Control mechanisms intervening within the transcriptional level are subdivided into two groups. While chromatin reorganizing factors control general chromatin accessibility by unpacking condensed, inaccessible DNA present as heterochromatin into accessible euchromatin, other regulatory proteins termed transcription factors directly bind DNA motives, influencing the expression of the according genes [150, 151]. Post-transcriptional regulators, like RNA binding proteins or miRNAs determine the fate of the mRNA in a promoting or inhibitory way [152, 153]. Furthermore, alternative splicing partially alters the information encoded on the mRNA resulting in differential protein outcomes. Post-translational regulatory factors may influence protein functionality by subjecting them to proteasome degradation through specific ubiquitination for example (**Figure 8**) [154].

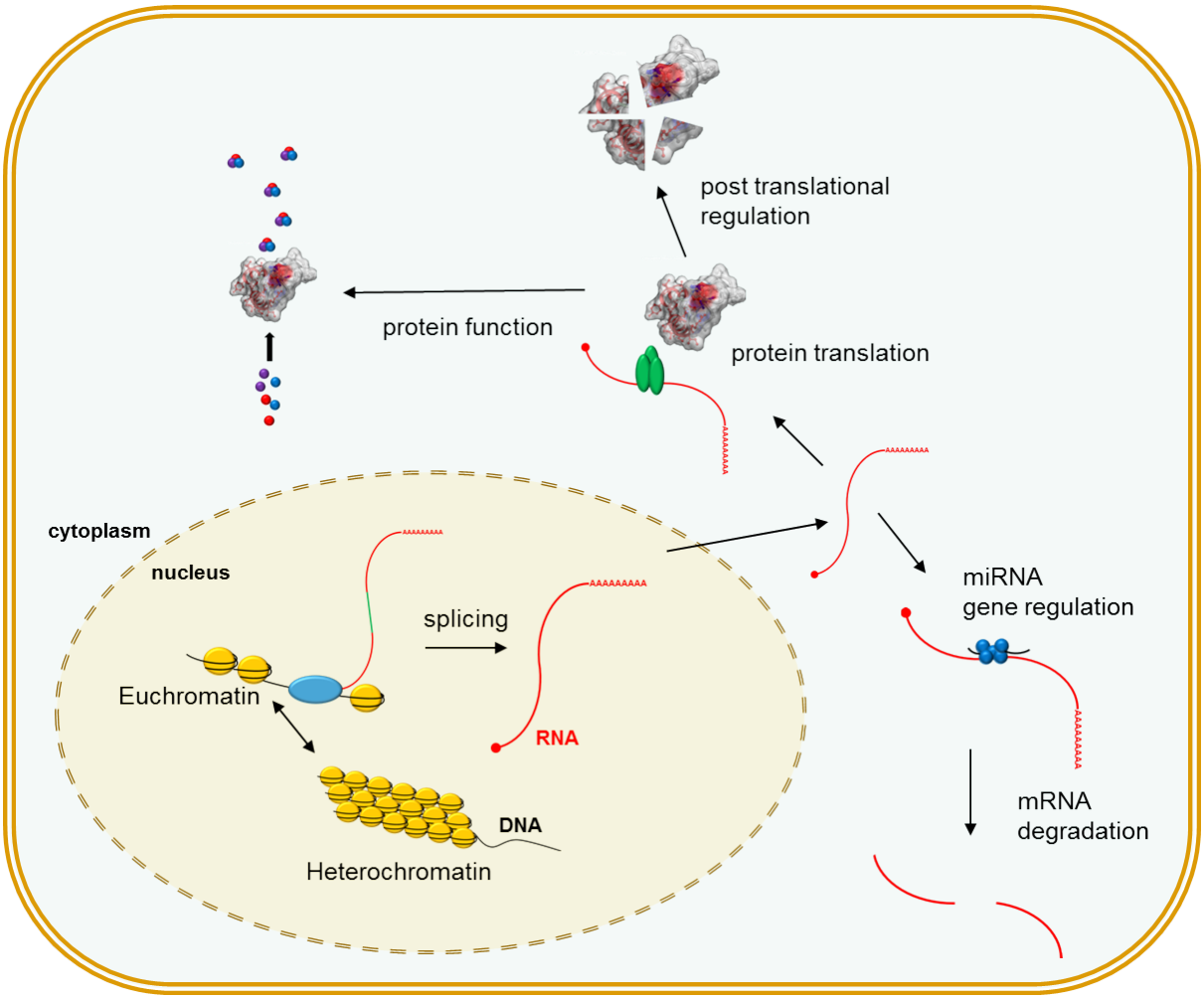

Figure 8: Regulatory mechanisms of gene expression – a schematic overview.

3.3.1 MicroRNAs

3.3.1.1 Overview

microRNAs (miRNAs) are a relatively novel class of small non-coding RNA molecules of about 22 nucleotides in length. By interfering with transcripts they play vital roles in posttranscriptional control of most mammalian genes. miRNA deregulation is associated with a variety of diseases, ranging from myocardial infarction to autoimmune disease and cancer [155].

3.3.1.2 Discovery and history

The fundamentals leading to the discovery of the first miRNAs were laid in *Caenorhabditis elegans (C.elegans)* in the early 1990s by Ambros and colleagues [156]. The small nematode

worm possesses two genes, namely lin-4 and lin-14, vital for larval development. Animals harboring null-mutations in the lin-4 gene exhibit severe developmental defects. Interestingly, simultaneous deactivated lin-14 reverts the malformed phenotype concluding a negative regulatory role of lin-4 against lin-14 [156]. The following search for typical gene features, such as start/stop codon, mRNA or even a translation product, remained unsuccessful. Furthermore, artificially introduced mutations in lin-4's putative open reading frame (ORF) could not jam the gene's function, indicating that lin-4 is anything else, but a regular gene. During the investigations, researchers encountered conspicuous short non-coding lin-4 transcripts of unknown function. One of these transcripts has a length of approximately 22 nucleotides and exhibits characteristic complementary sequence matches within the 3'UTR of lin-14's mRNA. A novel regulatory mechanism involving a small non-coding RNA was discovered [156, 157].

Until the discovery of the second miRNA, let-7, miRNAs were thought to be a phenomenon specific to *C.elegans*. Subsequently, let-7 family members and other miRNAs were found to be abundant in both invertebrates and vertebrates, including humans [158, 159]. Some of the miRNAs are highly conserved, suggesting that miRNA-mediated post-transcriptional regulation is a general regulatory function across species [158]. Today, thousands of miRNAs are known and their diverse functions in several biological aspects are gradually being discovered. Novel computer programs access large data bases and facilitate target prediction, which is essential for deciphering miRNA functionality [160].

3.3.1.3 Biogenesis

Most of the miRNA are encoded in exons/introns of protein-coding or non-coding genes, using them as so called "host genes" [161-163].

microRNA biogenesis is a complex multistep process starting with a regular transcription by RNA polymerase II in the nucleus [164]. The initial transcript shows mRNA features, like a 5' cap and a 3' poly-A tail, and forms one or multiple distinct secondary structure known as the pri-miRNA containing the miRNA stem loops [165]. DGCR8 or "Pasha" in concert with Drosha cleaves off the individual hairpins which are referred to as pre-miRNAs and exported into the cytoplasm by Exportin 5. Dicer recognizes the pre-miRNAs in the cytoplasm and cleaves off the joining loop as well as overhangs on the opposite site, leaving two imperfectly matched mature miRNAs of around 22 nucleotides each behind [166, 167]. In addition to that, a non-

canonic biogenesis pathway is known, where the pri-miRNA is processed by the spliceosome before nuclear export, circumventing the requirement for Drosha-mediated digestion in the nucleus (**Figure 9**) [168].

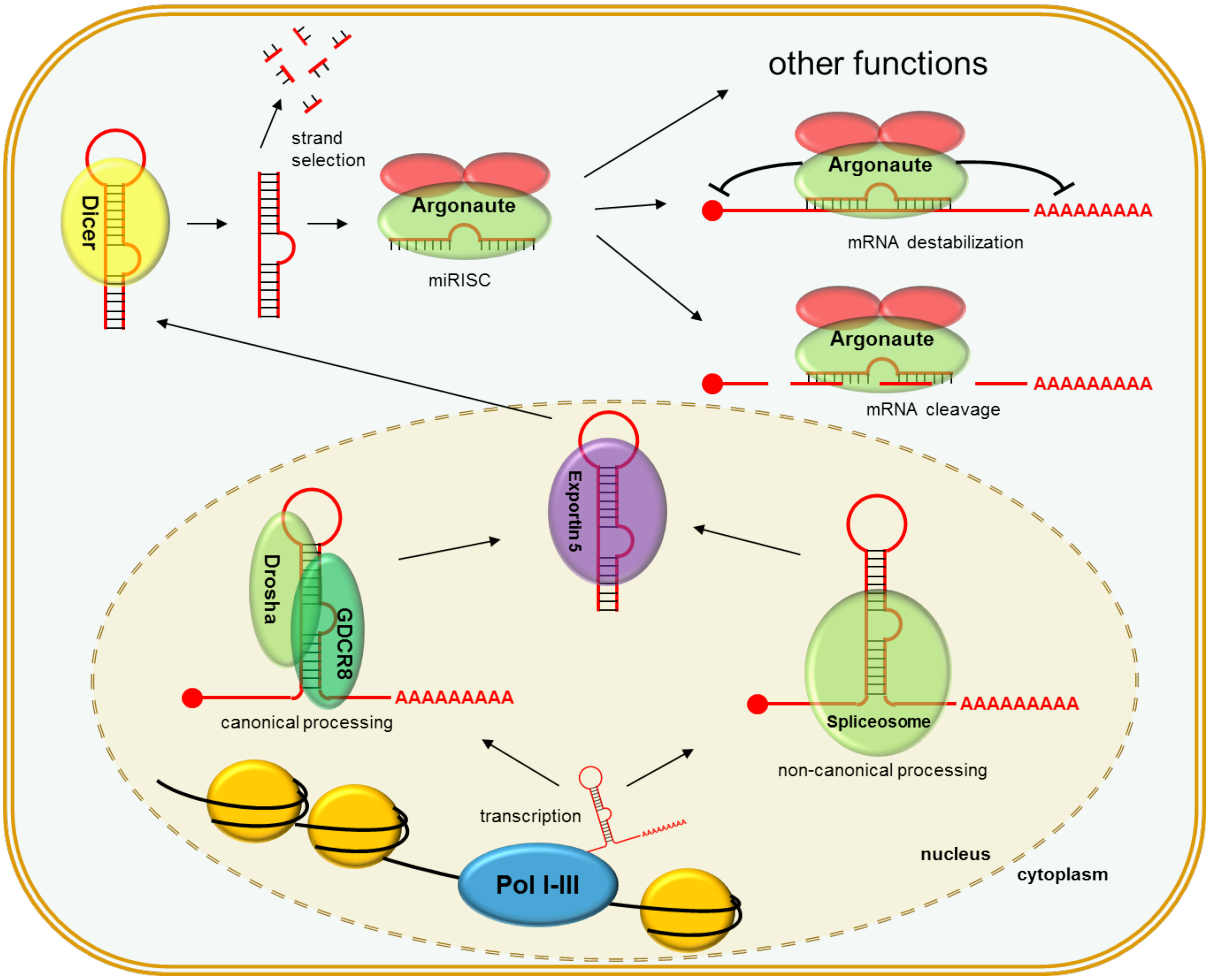

Figure 9: Schematic illustration of canonical and non-canonical miRNA biogenesis.

3.3.1.4 Nomenclature

Traditionally, miRNAs are named according to their discovery in a simple sequential way. A letter after the miRNA number differentiates among multiple members of the same miRNA family. In the case of a clear predominance of one mature miRNA strand, the short-lived passenger strand is labeled with an asterisk. If none of the strands is predominantly degraded or RISC incorporated, they are named according to their pre-miRNA position as 3-prime or 5-prime (3p or 5p). Mature miRNAs with identical sequence but different originating gene loci are suffixed with an additional number in the end. The species is indicated by a three letter abbreviation at the very beginning (**Figure 10**) [169].

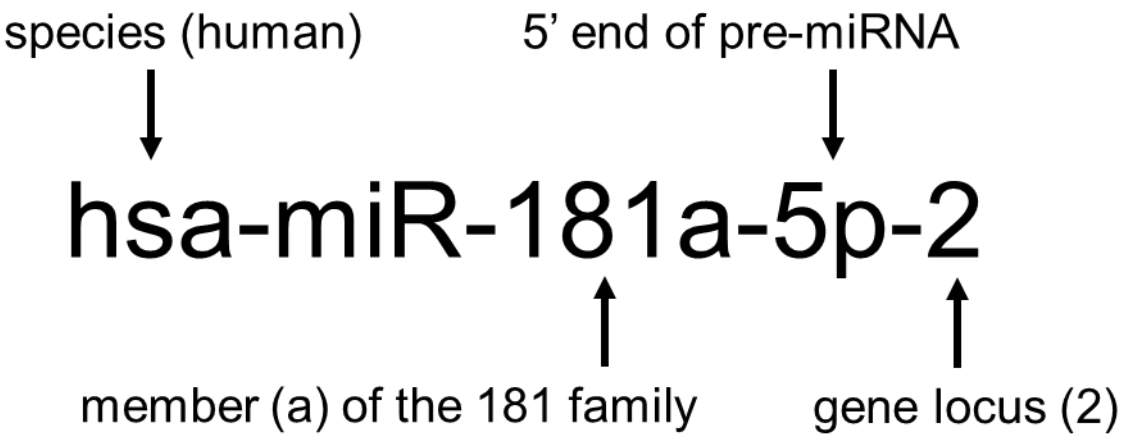

Figure 10: Standardized miRNA nomenclature. miRbase.org and [169].

3.3.1.5 Function

In general, miRNAs interact with the 3’UTR of their target mRNAs in a sequence-specific manner. Function-determining elements of this interaction are the seed region (usually nucleotide 2 – 8 seen from the 5’ end) and single matches further downstream towards the 3’ end of the miRNA. While sequence matching within the seed regions is rather stringent, downstream matches are thought to stabilize the miRNA – mRNA complex [170-172]. Interactions noncompliant with those rules like mismatches within the seed region, target sites within 5’UTR or coding region of the mRNA exist and are referred to as non-canonical binding sites. They typically require additional sequence matching within miRNA regions towards the 3’ end to efficiently repress protein translation [173, 174]. Other factors important in determining targeting efficacy include the length of the 3’ UTR, the site position within the 3’ UTR, closely spaced miRNA binding sites, seed-pairing stability of the miRNA to its target, accessibility of the site, and the target-site abundance of competing binding sites in the transcriptome. Due to the imperfection of miRNA – mRNA interactions, a miRNA is capable of targeting multiple mRNAs, and vice versa, a mRNA can be targeted by multiple miRNAs [175-177].

The mechanistic modes used by miRNAs to negatively regulate gene expression are versatile, highly complex and partially contradictory. In a huge effort undertaken by Morozova and colleagues, several established means of miRNA mediated gene regulation (Cap-40S initiation inhibition, 60S ribosomal unit joining inhibition, elongation inhibition, ribosome drop-off, co-translational nascent protein degradation, sequestration in P-bodies, mRNA destabilization, mRNA cleavage and transcriptional inhibition through microRNA-mediated chromatin

reorganization followed by gene silencing (summarized and reviewed in [178])) have been evaluated for their plausibility. Furthermore, they took advantage of a sophisticated computer algorithm, allowing them to estimate the predominant mode of action of a particular miRNA which is dictated by the relationships among the intrinsic characteristics of its target mRNA [179]. In some instances, miRNAs are even reported to be involved in histone modification and may even bind to promoters, enhancing gene expression [180, 181].

These circumstances result in versatile, tissue and context specific miRNA functionality, making miRNA research a highly complex subject.

3.3.1.5.1 RNA induced silencing complex

miRNAs require a complex protein aggregate for target guidance and execution of their versatile functions. The RNA induced silencing complex (RISC) is a multi-protein complex, more specifically a ribonucleoprotein, fundamental to miRNA function in mammalian cells [178]. Based on processes not yet fully understood, usually one mature miRNA strand is incorporated into the RISC while the other one, called passenger strand, is degraded [182]. Recent estimations suggest thermodynamic properties of a two nucleotide overhang at the miRNA 5' end to be sensed by members of RISC leading to guide strand selection [183]. The incorporated miRNA strand serves RISC as a template for target identification. Thereupon, various miRNA gene silencing modes, subdivided into mRNA degradation and inhibition of mRNA translation, are initiated [172].

Argonaute (Ago) family members, especially Ago 2 in mammals, are key players within the RISC complex, directly interacting with the incorporated miRNA and are, among other jobs, responsible for target mRNA cleavage [184]. This form of negative gene expression regulation is typically found in instances where a near perfect miRNA – mRNA match in present. Mainly for lose matches RISC is known to accelerate deadenylation and decapping, drastically decreasing mRNA stability and thereby protein expression [172, 185]. Debatably, RISC incorporated miRNAs can interfere with mRNAs mechanically, terminating or hindering protein translation [186]. Interestingly, some RISC independent mechanisms have been described, in which miRNAs achieve translational expression by spatially separating the target mRNA from the required translational components [187]. A comprehensive review Pratt and MacRae summarized our current understanding of how RISC mediates gene silencing [188].

3.3.1.5.2 Processing bodies

Distinct foci, microscopically appearing as small granules within the cytoplasm of eurcaryotic cells are called Processing Bodies (P-Bodies) (sometimes termed GW Bodies in mammalian cells) and are associated with mRNA turnover [189]. Since most of the proteins essential for miRNA functionality, like decapping or deadenylation enzymes, are found within these cellular organelles it is estimated that most of the miRNA processes are taking place there [190-192]. Next to miRNA induced silencing other mRNA decaying mechanisms, such as nonsense-mediated mRNA decay, adenylate-uridylate-rich element mediated mRNA decay, are associated with P-Bodies [193]. Furthermore, mRNAs are not necessarily degraded with the P-Body but can be stored and reenter translation at a given time [194]. The exact mechanisms, however, forming the basis of P-Body management are currently being investigated.

3.3.1.6 miRNAs and cancer

As indicated above, miRNAs play vital roles in several biological aspects. Little wonder that flaws in this substantial regulatory machinery lead to various diseases and cancer [155, 195]. According to their oncogenic or tumor suppressive role, miRNAs are referred to as oncomirs or tumor suppressor miRNAs respectively [196]. Their deregulation can lead to imbalanced cellular homeostasis resulting in cancerous behavior with increased proliferation or formation of metastasis for example. Indeed, a deregulated miRNA pattern has been observed many different cancers with impacts on several cancer hallmarks. Although miRNAs are considered as gene expression fine tuners, the deregulation of a single miRNA may lead to cancer in some instances [197, 198]. Along these lines, modulation of miRNA levels or in other words reestablishing miRNA regulation, has achieved cancer remission in a variety of in vitro and in vivo experiments. Furthermore, an increasing number of studies have identified miRNA candidates as useful biomarkers, allowing diagnosis and prognosis of various human diseases [199].

3.3.1.7 miR-181a

miR-181a is transcribed from two genes (*MIR181A1HG* and *MIR181A2HG*) located on Chromosome 2 and 9 [200]. Next to being involved into general cellular processes, this

particular miRNA candidate is known to be a key player in various cancers. Its role comprises involvement in many cancer hallmarks as an oncomir as well as a tumor suppressive miRNA. This discrepancy highlights the distinct tissue specificity of miRNAs in physiological processes [201].

3.3.1.8 miRNA target identification

As mentioned above, miRNA – mRNA interactions are highly context specific and need to be evaluated in each cell type of interest. First clues in the painstaking search can be drawn from bioinformatics predictions. Complex algorithms based on binding energies of miRNA – mRNA sequence matching depict several possible interactions, usually spitting out thousands of possible targets [202]. The search can be refined using databases like miRTarBase (mirtarbase.mbc.nctu.edu.tw), which hold a comprehensive collection of validated molecule interactions, sorted according to the validation method [203, 204].

Most experimental methods are based on artificial miRNA modulation and subsequent quantification of either multiple mRNA expression changes, via a gene arrays or mRNA sequencing, or single expression changes, using pPCR or Western blotting [205]. Highly sophisticated, and accordingly expensive techniques are Ago pulldown assays where Ago is linked to its incorporated miRNA and target and isolated, followed by identification via sequencing [206, 207].

Finally, the direct interaction between the miRNA and the 3'UTR of its target mRNA has to be proven. The gold standard comprises a dual luciferase plasmid, transfected into the cell. miRNA interaction with the according 3'UTR, which is part of the construct, will prevent translation of the downstream luciferase gene. The degree of translational repression can be quantified photometrically [208, 209]. In our study, we used a *KRAS* overexpressing plasmid as a backbone and cloned the 3'UTR of interest upstream of the start codon. Thereby *KRAS* protein expression and the corresponding function can be read out simultaneously proving a direct link between the miRNA, the target 3'UTR and the functional consequence.

3.3.1.9 miRNA therapy options

By regulating the majority of the human transcriptome, miRNAs play important roles in development, homeostasis and disease [155]. Treatments against inherited diseases, so called gene therapies, are extremely challenging. Due to the precise spatiotemporal regulation of miRNAs during development, these challenges reach another dimension of complexity, supposedly provoking more damage than benefits [210]. miRNA therapies against diseases of the adult body however, are much more promising as backed up by examples in hepatitis C Virus infection, insulin resistance and cardio-metabolic disease [122, 211-213].

Similarly, as in research, most of the therapeutic approaches make use of small DNA/RNA molecules or their derivatives interfering with endogenous miRNAs by partial or complete sequence complementarity. Thereby, the pathologic miRNA is either subjected to degradation or "sponged/trapped" leading to its inactivation in either case [214-216].

The reverse approach is the therapeutic application of miRNA mimics, reestablishing pathologically low miRNA levels or targeting critical onco genes for example [217]. This strategy came to use successfully against cancers using let-7 as a template. miR-34 mimics are currently in clinical Phase I trials for treatment of metastasized cancer involving the liver [122].

Therapeutic small nucleotide molecules are well elaborated constructs containing chemical modifications and sometimes DNA/RNA adducts further improving their stability [218]. Still, miRNA inhibitors are easier to design than most small molecule inhibitors, because their specificity can be predicted and modified following the rules of Watson-Crick base pairing. Furthermore, miRNAs are, in contrast to small molecule inhibitors or protein based drug molecules, physiologic antisense nucleotides causing less side effects, like toxicity or excessive inflammation [122].

The main challenge however, remains efficient and reliable delivery of the miRNA cargo to its destination. Huge leaps forward have been recently made by the development of nanoparticles and liposomes in targeting solid tumors in mouse experiments via simple injection into blood vessels. Enhanced permeability and retention effects, arising from pathophysiological abnormalities encountered in solid cancers, facilitate passive delivery of these nanocarriers [219]. More specific miRNA delivery is achieved by linking miRNAs to different types of moieties, such as cholesterol [218], α-tocopherol [220], CpG-containing oligonucleotides [221], or antibody fragments [222]. The vast majority of these strategies however, aims at organs with central positions within the blood circulation, like liver, lung or

kidneys and therefore easy accessibility for systematic treatments. Organs like the skin, in contrast, cannot be targeted by those means, since most of the miRNA cargo would be trapped in the liver [223]. Extensive efforts are currently undertaken to facilitate miRNA delivery into distinct skin regions by refining cream-like formulations for topical skin treatment [224-226].

4. Aim of the Thesis

Our group holds a strong research interest in molecular processes underlying formation and progression of SCC particularly in regard to laying fundamentals for novel therapeutic options. Continuous research efforts resulted in huge leaps in the field of efficient miRNA delivery and paved the way for the first miRNA therapies against cancer that are now starting to emerge. Inspired by the success of these therapies in early clinical trials and the accessibility of the skin for topical treatments we were prompted to find out more about the role of miRNAs, miR-181a particularly, in SCC. Due to the complex nature of miRNA functionality, these small molecular regulators have highly variable functions depending on the cellular environments. For many miRNA candidates, completely opposing outcomes have been observed depending on the organ in which they are acting. Most of the miR-181a studies in cancer, attest miR-181a a cancer promoting role, by accelerating general cancer aspects, such as proliferation. The very same miRNA has been shown to inhibit formation of metastasis in other cancers. To date, little is known about miRNAs in SCC in general and no report so far investigated the specific role of miR-181a in SCC.

Combating SCC's tendency of invading into dermal layers and forming distant metastasis makes a detailed knowledge of the responsible molecular mechanistic essential. Based on studies in other epithelial cancers, we were aiming to unravel *CYFIP1's* role as a negative regulator of keratinocyte invasion. Furthermore, *CYFIP1* is under direct *NOTCH1* control suggesting a molecular coherence in SCCs that induces cellular differentiation and arrests cellular invasion. This study could be of high clinical significance, as it suggests a rationale for the relationship between SCC differentiation status and its invasive potential.

5. Materials and Methods

5.1 **Materials**

5.1.1 Cell culture

SCC12 cell line:
originally isolated from SCC of facial skin of a 60-year old male kidney-transplant recipient [227].

SCC13 cell line:
originally isolated from SCC of facial skin of a 56-year old female [227].

HaCaT cell line:
spontaneously immortalized keratinocyte cell line, originally isolated from the back skin of a 62-year old male [228].

HEK293T cell line:
Originally are isolated from human embryonic kidneys (HEK). The 293T cells are transformed with large T antigen [229].

Primary keratinocytes:
Isolated from healthy skin obtained from mammary and abdominal surgery at the University Hospital Zurich.

Primary SCC cells:
Isolated from SCC specimen, excised at the Department of Dermatology at the University Hospital Zurich.

5.1.2 Animals

Female nude mice (Hsd:AthymicNude-Foxn1<nu>) Harlan Laboratories Inc.

5.1.3 Reagents

Agarose	Sigma (A9539)
Ampicilin	Santa Cruz Biotechnology (210812)
Antibiotic-Antimycotic (100x)	Gibco® (15240-096)
Blasticin	InvivoGen (ant-bl-1)
Bovine Pituitary Extract (BPE)	Gibco® (37000-015)
CnT07 and basal medium	CELLnTEC (CnT-07)
Doxycycline	Sigma (D9891)
Doxycycline, *in vivo* application	Bioserv (S3888)
Dulbecco's Modified Eagle Medium (DMEM)	Gibco® (41966052)
Epidermal Growth Factor 1-53 (EGF 1-53)	Gibco® (37000-015)
Fetal Bovine Serum Gold PAA	VWR: 95025-540
G418 Gentamycine	Roche (04727878001)
Kanamycine	Sigma (70560-51-9)
Keratinocyte serum-free medium (K-SFM)	Gibco® (10744-019)
LB Agar	Scharlau (01-385-500)
LB Agar (instant mixture)	InvivoGen (fas-bl-s)
LB Broth	Scharlau (02-384-500)
NuPAGE LDS Sample Buffer	novex (NP0007)
NuPAGE Sample Reducing Agents	novex (NP0009)
PBS Dulbecco without calcium and magnesium	Gibco® (10010-015)
PMSF	Sigma (P7626)
Puromycin	Santa Cruz Biotechnology (108071)
RIpa Buffer	Cell Signaling Technology (#9806)
TB liquid (instant mixture)	InvivoGen (fas-bl-1)
Trypsin/EDTA Solution	Merck Millipore (SM-2003)
Vaseline	BAYER (85403-1EA)

Transfection reagents	
Gene Carrier-1™	Epoche (31-00110)
INTERFERin	Polyplus (409-50)

5.1.4 DNA, RNA and derivatives

5.1.4.1 miRNA mimics and inhibitors

mirVana mimic control #1	Ambion (4464058)
mirVana miR-181a mimics	Ambion (4464066)
mirVana inhibitor control #1	Ambion (4464076)
mirVana miR-181a inhibitors	Ambion (4464084)

5.1.4.2 Synthetic constructs

CYFIP1 putative promotor region	Blue Heron (custom made)
gBlocks miR-181a-1	IDT (custom made)
gBlocks miR-181a-2	IDT (custom made)
gBlocks Notch1 intracellular domain	IDT (custom made)

5.1.4.3 siRNA sequences

Qiagen

control sequence: 5’ – AATTCTCCGAACGTGTCACGT – 3’

siKRAS_7: 5’ – CTCCTAATTATTGTAATGTAA – 3’

siKRAS_8: 5’ – AAGGAGAATTTAATAAAGATA – 3’

CYFIP1_1: upon request

CYFIP1_2: upon request

5.1.4.4 Primer sequences

Microsynth AG

36B4 (housekeeping gene)

Forward: 5’ - GCAATGTTGCCAGTGTCT - 3’

Reverse: 5’ - GCCTTGACCTTTTCAGCA - 3’

CYFIP1

Forward: 5’ - CTGCACGCGGCTCCTTTCCA - 3’

Reverse: 5’- GACAAGATGCAGCGGGGCGT -3’

CYFIP1 promotor region (-2.9k bp)

Forward: 5’ – TAGAGTGTGCTACTATCTGTC - 3’

Reverse: 5’ – ATGAAGGTTTGGTTACCCCC - 3’

CYFIP1 promotor region (-0.9k bp)

Forward: 5’ - ATCCAAAGCCCCTGTTTTGC - 3’

Reverse: 5’ – GTCTCATCAGATTTCAAAGGG - 3’

Filaggrin

Forward: 5’ - TGAAGCCTATGACACCAC - 3’

Reverse: 5’ - TCCCCTACGCTTTCTTGT - 3’

HES1:

Forward: 5’- GGTGCTGATAACAGCGGAAT - 3‘

Reverse: 5’- TGAGCAAGTGCTGAGGGTTT - 3’

HES1 promotor region

Forward: 5’ – CCTCCCATTGGCTGAAAGTT - 3’

Reverse: 5’ – CCTGGCGGCCTCTATATATA - 3’

Involucrin

Forward: 5’ - TCCTCCAGTCAATACCCA - 3’

Reverse: 5’ - CAGCAGTCATGTGCTTTT - 3’

Keratin 10

Forward: 5’ - GGTGGGAGTTATGGAGGCAG - 3’

Reverse: 5’ - CGAACTTTGTCCAAGTAGGAAGC - 3’

KRAS

Forward: 5’ - AAGACAGAGAGTGGAGGATGC - 3’

Reverse: 5’ - GTGCTGAACTTAAACTTACCAGAT - 3’

Loricrin

Forward: 5’ - GCGAAGGAGTTGGAG - 3’

Reverse: 5’ - CTGGGTTGGGAGGTACT - 3’

pIND20 Notch1

Forward: 5’ - CCGCTACTCACGCTCTGA - 3’

Reverse: 5’ - CTGGAAGACACCTTGTGC - 3’

5.1.4.5 Mutagenesis primer sequences

Microsynth AG

Binding site 1:

forward

5’ - CTTCTTATTTTTCTTACCAATTGTTCCGGTTGGTGTGAAACAAATTAATGAAGC - 3’

reverse

5’ - GCTTCATTAATTTGTTTCACACCAACCGGAACAATTGGTAAGAAAAATAAGAAG - 3’

Binding site 2:

forward

5’ - GTCCTATAGTTTGTCATCCCTGATTCCGGTAAAGTTACACTGTTCACAAAGGTTTTGTC - 3’

reverse

5’ - GACAAAACCTTTGTGAACAGTGTAACTTTACCGGAATCAGGGATGACAAACTATAGGAC - 3’

Binding site 3:

forward

5’ - CGTATATTGTATCATTTGAGTTCCGGTTCCCAAGTAGGCATTCTAGGC - 3’

reverse

5’ - GCCTAGAATGCCTACTTGGGAACCGGAACTCAAATGATACAATATACG - 3’

5.1.4.6 Plasmids and vectors

nNERT-neo	U Just, Kiel
pcDNA3	GP Dotto, Lausanne
pcDNA3/hNIC	GP Dotto, Lausanne
pcDNA3-Notch1	GP Dotto, Lausanne
pGL3-basic	Promega
pGL4.75-KRAS LCS6m	Addgene Plasmid #44571
phRL-TK	GP Dotto, Lausanne
phRL-TK	Promega
pincoGFP	GP Dotto, Lausanne
pMD2.G	Addgene Plasmid # 12259
psPAX2	Addgene Plasmid # 12260
pTRIPZ	GE Healthcare
pUNO1 KRAS	Sigma
RBPjk-luc plasmid	Promega
rNeo	U Just, Kiel

5.1.4.7 Viral particles

MISSION® Lenti microRNA Inhibitors control #1	Sigma (HLTUD001C)
MISSION® Lenti microRNA control #1	Sigma (NCLMIR001)
MISSION® Lenti miR-181a Inhibitors	Sigma (custom made)
MISSION® Lenti miR-181a	Sigma (custom made)

5.1.5 Antibodies

5.1.5.1 Primary antibodies

Antibody	Dilution	Supplier
Anti-ERK	1:1000	Cell Signaling (#9102)
Anti-p-ERK	1:1000	Cell Signaling (#9101)

Anti-KRAS	1:200	Santa Cruz Bio. (sc-30)
Anti-filaggrin	1:200	Santa Cruz Bio. (sc-66192)
Anti-involucrin	1:200	Santa Cruz Bio. (sc-21748)
Anti-loricrin	1:200	Santa Cruz Bio. (sc-51130)
Anti-CYFIP1	1:1000	Merk-Millipore (07-531)
Anti-actin	1:1000	Santa Cruz Bio. (sc-47778)
Anti-Notch1	1:200	Santa Cruz Bio. (sc-6014)
Anti-p21	1:200	Santa Cruz Bio. (sc-6246)
Anti-Hes1	1:1000	Merk-Millipore (AB5702)

5.1.5.2 Secondary antibodies

Antibody	Dilution	Supplier
Anti-rabbit HRP	1:5.000	Cell Signaling (#7074)
Anti-mouse HRP	1:10.000	Abcam (ab6728)
Anti-goat HRP	1:8.000	Santa Cruz Bio. (sc-2768)
Anti-mouse IRDye® 680LT	1:10.000	LI-COR (926-68022)
Anti-mouse IRDye® 800CW	1:10.000	LI-COR (925-32212)
Anti-rabbit IRDye® 680LT	1:10.000	LI-COR (926-68023)
Anti-rabbit IRDye® 800CW	1:10.000	LI-COR (925-32213)
Anti-goat IRDye® 680LT	1:10.000	LI-COR (926-68024)
Anti-goat IRDye® 800CW	1:10.000	LI-COR (925-32214)
Anti-rabbit FITC conjugated	1:25	Dako (F005401-2)

5.1.6 Cloning enzymes

Alkaline phosphatase	NEB (M0290S)
BamHI	NEB (R0136S)
BglII	NEB (R0144S)

EcoRI	NEB (R0101S)
KpnI	NEB (R0142S)
Nhel-HF	NEB (R3131S)
Phusion-HF polymerase	NEB (M0530S)
T4 DNA ligase	NEB (M0202S)
XhoI	NEB (R0146S)

5.1.7 Kits

BioCoat Matrigel Invasion Chambers	BD (354480)
BrdU assy kit	Millipore (2752)
Dc Protein Assay	BioRad (500-0116)
Diff Quick	Medion Diagnostics (130832)
Dual Luciferase assay reporter kit	Promega (E1910)
FITC Annexin V Apoptosis Detection Kit I	BD (556547)
Gel DNA Recovery Kit	Zymoclean (D4001)
Genopure Plasmid Maxi Kit	Roche (03143422001)
Gibson Assembly® Cloning Kit	NEB (E5510S)
GoScript™ Reverse Transcription System	Promega (A5000)
High Pure Plasmid Isolation Kit	Roche (11754277001)
REAL Detection System (APAAP kit)	Dako (K5005)
RNeasy® MiniElute cleanup Kit	Qiagen (74204)
TaqMan microRNA assay (miR-181a)	Life Technologies (4427975-480)
TaqMan microRNA assay (Z30)	Life Technologies (4427975-1092)

5.1.8 Chemicals

Bovine serum albumin (BSA)	Sigma (A9418)
Chloroform	Sigma (472476)
Citric acid	Sigma (251275)
Dimethyl sulfoxide (DMSO)	Sigma (D4540)
Dispase II	Roche (04942078001)

ECL solution	Amersham (RPN2106)
Ethanol	Sigma (02860)
FastStart Universal SYBR Green Master (ROX)	Roche (04913914001)
Formaldehyd (4%, buffered)	Kantonsapotheke Zurich
Formic acid	Sigma (F0507)
Glycerol	Fluka (49783)
Glycine	Sigma (G7126)
Hexadimethrine bromide	Sigma (H9268)
Hydrochloric acid (HCl)	Fluka (84415)
Isopropanol	Fluka (34965)
Methanol	Sigma (32213)
Protein ladder	Geneaid (PL0245)
Saponin	Sigma (8047-15-2)
Sodium chloride	Sigma (S9625)
Sodium dodecyl sulfat (SDS)	Sigma (71725)
TritonX100	Thermo Scientific (85112)
Trizma® base	Sigma (T1503)
TRIzol® Reagent	Ambion® (15596018)
Tween 20	Sigma (93773)

5.1.9 Buffers

10x SDS-PAGE running buffer

144 g Glycine

30 g Trizma® base

50 ml 20%SDS

Add H2O to 1 l

use: 100 ml 10x Running buffer + 900 ml H2O

10x TBS

60.6 g Trizma® base

87.6 g Sodium chloride

Adjust pH to 7.6 with HCl

Add H2O to 1 l

10x Transfer buffer

144 g Glycine

30 g Trizma® base

Add H2O to 1 l

use: 100 ml 10x Transfer buffer + 800 ml H2O + 100 ml Methanol

RIPA buffer Cell Signaling (#9806)

+ 1mM PMSF before use

Stripping buffer

15 g glycine

1 g SDS

10 ml Tween 20

Adjust pH to 2.2 with HCl

Add H2O to 1 l

TBS/T (washing buffer for WB)

100 ml 10x TBS

900 ml H2O

0.1% Tween 20 (1 ml)

5.1.10 Consumables

Cell culture dishes	Falcon
Cell culture flasks	Falcon
Cell culture well plates	Falcon
Cloning cylinders	Sigma (C3983-50A)
Combitips	Eppendorf
Cover slips (18x18 mm)	Menzel GmbH (BB018018A1)
Cryo tubes Nalgene	VWR International AG

Eppendorf tube (1.5 ml, 2 ml)	Eppendorf
Falcon tubes	Corning Life Science
Glass slides Superfrost Plus	Thermo Scientific (J1810AMNZ)
Hyperfilm ECL	Amersham Bioscience (28-9068)
MicroAmp® Optical 96-Well Reaction Plate	Applied Biosystems
Nitrocellulose membrane	Amersham Bioscience (10600002)
PVDF membrane	BioRad (162-0177)
RNase inhibitor (RNase ZAP)	MBP (7002)
Scalpels	Swann-Morton (0208)
Whatman paper	Whatman (514-8013/3030917)

5.1.11 Devices

FACSCanto	BD Biosciences
Film developer	Thermo Fisher Scientific
Microscope CLSM Leica SP5	Leica
Mini Protean® 3 Cell (electrophoresis system)	BioRad
Mini Trans-Blot® Cell (transfer system)	BioRad
Plate reader	Tecan
Power supply	BioRad
ScanScope	Aperio
ViiA7 qPCR machine	Applied Biosystems

5.1.12 Software

Adobe Acrobat XI Pro	Adobe
Adobe Illustrator CS6	Adobe
Endnote X6 Thomson	Reuters
FlowJo	Ashland
Gene Ape	biologylabs
GraphPad Prism 5	GraphPad Software
Image J 1.47t	National Institute of Health
Image Studio™ Lite	LICOR
Microsoft office excel 2010	Microsoft

ScanScope Aperio

5.2 Methods

Experiments were conducted in a bio safety level 2 (BSL2) laboratory according to BAFU standards. All procedures were carried out at room temperature unless otherwise stated.

5.2.1 Cell culture

General maintenance

HaCaT, SCC13 and HEK293T were cultured in DMEM high glucose supplemented with 10% FCS. Gibco's SMF Keratinocyte medium, supplemented with EGF and Bovine Pituitary Extract (BPE), was used for SCC12. Primary patient-derived cells were grown in CnT-07 medium and the enclosed supplements. Cells were split after reaching a confluence of 80% to 90% using trypsin.

Several cells were cultivated in a humidified 5% CO_2 atmosphere at 37°C.

Temporally unused cell lines, were stored at -80°C in freezing medium (FBS supplemented with 10% DMSO).

Institutional board approval for the use of human tissue was granted; all donors signed written informed consent forms in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans (ethical approval number EK647). All samples were obtained from the University Hospital Zurich (Zurich, Switzerland).

5.2.2 Generation of primary patient derived cell cultures

Left over skin from mammary or abdominal reduction surgeries was collected at the University Hospital Zürich after patients signed consent forms mentioned above. Using a scalpel, the fat tissue was removed from the dermis and discarded. Dermal parts were washed in PBS several times and cut into 0.5 – 1 cm^2 squared pieces. SCC material was biopsied from excised SCC parts not needed for histological diagnosis. By incubating preparations in CnT-07 medium containing antibiotics and 10 mg/ml Dispase II at 4°C overnight, the basal lamina was digested, allowing separation of dermis and epidermis. The obtained epidermal squares were incubated

us side down in trypsin for 10 minutes at room temperature followed by rigorous stirring, using tweezers, in order to bring keratinocytes into suspension. This step was repeated three times in CnT-07 medium. Cell suspensions were pelleted in a standard centrifuge, resuspended in fresh full medium, containing antibiotics, and cultured in cell culture flasks.

5.2.3 Viability and proliferation assays

5.2.3.1 WST-1 assay

2000 – 3000 treated cells/well were seeded into 96 well plates in the according growth medium. After 96 hours incubation time the medium was swapped with fresh one containing 1:10 WST-1 and 10% FCS and put back to 37°C. After a clear color change could be observed (approx. 60 minutes, depending on cell line) plates were subjected to photometric measurement at 450nm and 620nm reference measurement. Since values around 1 are desirable, the plate can be re-incubated on 37°C and re-measured at later time points to amplify low value readings.

5.2.3.2 BrdU proliferation assay

Effects of various treatments on cellular proliferation was assessed by bromodeoxyuridine (BrdU) incorporation. After initiation of the indicated treatment, cells were brought into suspension and seeded into the wells of a 96 well plate at a density of 2000 – 3000 cells/well in the according medium. After attachment, fresh medium containing BrdU reagent was added and incorporated for 96 hours followed by fixation. Immunologic BrdU detection and optical measurement was carried out as described in the manufacturer's protocol.

5.2.3.3 Manual cell counting

20000 – 30000 treated cells/ml were seeded into 3cm petri dishes in the according growth medium. After 96 hours incubation time pictures of 3 non-overlapping cell layer sections were taken and manually counted using imageJ.

5.2.4 Possibilities and limitations of miRNA research

Generally spoken, the working area and tools need to be RNAse free and proper sample cooling needs to be given [230].

miRNAs can be artificially upregulated in vitro and in vivo by different means. By transiently transfecting small chemically modified RNA/DNA molecules, increased miRNA presence can be simulated. Since the utmost part of the cargo gets stuck in membrane compartments or stays floating in the growth medium, the actual amount of active (or RISC incorporated) miRNA is hard to estimate [231]. Alternatively, constructs coding for miRNA precursors can be integrated in the cellular genome which are channeled into the endogenous miRNA processing machinery. This system allows for better fine tuning and can be easily used in vivo due to its high robustness [232]. The generation however, is much more work intensive and therefore connected to relatively high costs. miRNA knock down, can be achieved analogously, via transfection or stable integration of complementary DNA/RNA molecules which trap endogenous miRNAs and may additionally induce their rapid degradation [233].

Cellular miRNA levels are directly measured by TaqMan qPCR, however, during result interpretation special care needs to be taken. While pPCR determination of miRNA levels modulated by genomically integrated constructs is common practice, the method is considered unsuitable for modulations achieved by transfection of miRNA mimics. Results will mirror the total amount of miRNA mimics introduced into the experiment and not only the tiny fraction of interest, which are the Ago incorporated molecules [234]. A similar principal is true for transiently transfected miRNA inhibitors. During RNA extraction inhibitors and their targets are extracted simultaneously meaning that they can still interfere during the qPCR experiment. Hence the obtained results do not necessarily mirror the cellular miRNA levels. The qPCR based readout of a known miRNA target reflects the cellular situation much closer in these instances. Importantly, the tissue specificity of miRNA – mRNA interactions has to be taken into account when choosing a target gene for read outs [235].

5.2.4.1 RNA isolation

RNA was isolated form pelleted cells or tissue samples using TRIzol (Invitrogen) following the manufacturer's protocol. RNA quality was determined by photometry at 260/280 nm and 260/230 nm, whereas values above 1.8 were considered suitable. In case of poor quality RNA yield, an additional purification using Qiagen's RNA clean up kit could be performed.

5.2.4.2 Reverse transcription and SYBR green qPCR

Gene expression analyses of coding genes (i.e. long transcripts) was performed using the GoScript Reverse Transcription Kit (Promega) and SYBR Green (Roche). 1 µg of total RNA, transcribed into cDNA according to the manufacturer's manual using oligo dT primers and diluted 1:3 with H2O. Every well of the sub sequent qPCR reaction loaded with the following reagents:

10 µl FastStart Universal SYBR Green Master (Roche)

8 µl H_2O

0.5 µl primer solution (10 µM) in each direction

1 µl cDNA

Each reaction was carried out in quadruplicates alongside a cDNA free negative control and 36B4 as housekeeping gene. After pipetting, the plate was sealed using a plastic cover and centrifuged to remove air bubbles. The PCR reaction was carried out in a Viia7 real time PCR machine (AB Applied Biosystems) with the following setup: hold stage (95°C, 10 min), PCR stage (95°C, 10 sec; 60°C, 30 sec) repeat for 40 cycles, melt curve stage (95°C, 15 sec; 60°C, 1 min; 95°C, 15 sec). Relative gene expression was calculated by normalization to the house keeping gene and determining fold change of relative gene expression.

5.2.5 Reverse transcription and TaqMan qPCR

Short transcripts (i.e. miRNAs) on the other hand were analyzed using LifeTechnologies' TaqMan microRNA Assays. The kits implement a twostep procedure where miRNAs of interest are transcribed into cDNA using loped primers followed by common TaqMan qPCR. Small nuclear RNA (snRNA) Z30 was used as "Housekeeping gene" for normalization. All assays were performed according to the manufacturer's protocols.

5.2.6 Protein quantification

According to the manufacturer's protocol, reagents were incubated with the protein lysate for 30 minutes in the dark. After photometric read out at 690 nm wave length the protein content was calculated by comparing it to a standard curve.

5.2.7 Protein isolation

Cells were washed with ice cold PBS and lysed in RIPA buffer (Cell Signaling Technology) for 15 minutes followed by centrifugation at 16000xg at 4°C for 20 minutes. Supernatants were collected and quantified for protein content using BIO RAD's DC protein Assay and photometry at 690nm.

5.2.8 SDS PAGE and Western blotting

20 µg of protein were reduced in NuPAGE LDS Sample Buffer and Sample reducing agent by boiling at 95°C for 5 minutes. Samples were loaded on a NuPage 4 – 12% Bis-Tris gradient gel and put in MES SDS running buffer. The gel ran at a constant voltage of 150V for approx. one hour. After proteins were separated by size, they were blotted on a PVDF or nitrocellulose membrane using transfer Buffer containing 15% methanol. Therefore, a constant voltage of 100V was applied for 45 – 60 minutes, depending on the protein size of interest. After blotting the membrane was incubated in blocking buffer for 90 minutes followed by primary antibody incubation at 4°C overnight. Antibodies were used in BSA blocking buffer.

The next day membranes were washed three times for 10 minutes in washing buffer on a rocker followed by secondary antibody incubation for one hour. Antibodies were diluted in BSA blocking buffer.

Thereupon membranes were washed as stated above.
Proteins were detected using ECL Western Blotting Detection Reagent and chemiluminescence Hyperfilm and an automated developing machine following manufacturer's protocol.
Actin was generally used as a loading control and pan ERK during phospho ERK detection.

5.2.9 Flow cytometry

Quantification of apoptotic cells:
SCC13 cells, transfected with miR-181a mimics, were stained using BD Pharmigen's FITC Annexin V Apoptosis Detection Kit I following the manufacturer's protocol. UVB-irradiated cells were used as a positive control and to adjust proper gate setup. Another subset of cells

was boiled at 96°C for 5 minutes, representing dead cells and serving as an additional gating aide. Measurements were performed on a FACSCanto device. Data were analyzed with FlowJo software.

Quantification of CYFIP1 expressing cells:
Prior to staining, SCC 13 cells were fixed with 1% PFA and permeabilized with 1% saponin. For the assessment of the protein expression the following antibodies were used: unconjugated polyclonal rabbit anti-human CYFIP1 in the dilution 1:100 with FITC conjugated secondary swine anti-rabbit antibody, diluted 1:25. Measurements were performed on a FACSCanto device. Data were analyzed with FlowJo software.

5.2.10 Transfection

Transient transfection of siRNAs, miRNA mimics and miRNA inhibitors was performed using Interferin. Lager constructs, like DNA plasmids, were transfected by the aid of GeneCarrier 1. All transfections were carried out for 48 hours and following the manufacturer's protocol. In short, Cells were seeded 24 hours prior to transfection followed by medium change. The according transfection reagent was diluted in basal medium (cell culture medium without FCS or other supplements) after nucleotides were added. After 30 minutes of incubation the solution was added drop wise to the cells and incubated at 37°C for 48 hours.

5.2.11 Transduction

Lentiviral particles were either obtained by Sigma Aldrich (Mission Lentiviral particles) or produced using the psPAX2 Second Generation System in HEK293T cells following a modified protocol based on Barde et al [236]. psPAX2, pMD2.G and the construct to be packed were transfected into HEK293T cells in a weight ratio of 1:3:4. After 24 hours the transfection medium was swapped with DMEM 10% FCS for three consecutive days. All supernatants were collected and stored at 4°C in the dark. At the end of the production phase, all supernatants were pooled following slow centrifugation at 500xg to remove detached cells and cell debris. In order to pellet the lentiviral particles, the supernatant was ultra-centrifuged at 16°C, 50,000xg for 120 minutes. After resuspending pellets in 500 µl DMEM 10% FCS, viral stocks could be stored at -80°C or directly used. For the transduction procedure SCC13 cells were seeded at ~30% confluence into 10cm petri dishes 24 hours prior to the experiment. Medium

was swapped with DMEM 10% FCS containing 8µg/ml of hexadimethrine bromide and a 500 µl stock of resuspended lentiviral particles and incubated overnight at 37°C. The next day, medium was swapped with regular DMEM 10% FCS. After another 24 hours, the selection process was initiated using puromycin selection medium (DMEM 10% FCS supplemented with 5µM puromycin). The selection medium was changed daily until successfully transduced cells formed clones measuring approx. 3mm in diameter. By the use of cloning cylinders in combination with high viscosity Vaseline the cell clones were isolated, detached and transferred into 96 well plates. Cell clones were expanded and tested for functionality via qPCR or Western Blot.

5.2.12 In vivo tumor xenograft

All experiments were performed according to guidelines of the approved "Bundesamt für Lebensmittelsicherheit und Veterinärwesen - BLV" protocol. Female nude mice (4–6 weeks old) were ordered from *Harlan Laboratories Inc*. and acclimatized to the mouse facility for 10 days. HaCaT kd miR-181a, SCC13 Tet-On miR-181a or the according number of control cells were suspended in PBS to a final concentration of 4×10^7 or 1×10^7 per ml, respectively. 100 µl of the cell suspension were injected subcutaneously in one or both flanks using 0.5ml insulin syringes. Doxycycline for the Tet-On experiment was administered via food pellets (200mg/kg). The tumors were measured three times a week using a caliper. The tumor volume was calculated using the formula volume, $V = L \times W^2/2$, where L corresponded to long diameter and W to short diameter [237]. In parallel, mice where weighted once per week using a standard laboratory scale. The experiment was terminated after reaching significance. Single mice were euthanatized in case one or more of the termination criteria were fulfilled, such as tumor mass greater than $1cm^3$, ulcerating tumors, severe weight loss or lack of fight/flee instincts. Tumors of dead mice were dissected, whereat RNA was isolated from one half and hematoxylin-eosin (H&E) slides were produced from the other part.

5.2.13 Histology

5.2.13.1 Hematoxylin and eosin staining

Excised tumors from terminated mice were fixed in 4% formalin, followed by dehydration and embedding in paraffin. Seven micron sections were cut, deparafinized, dehydrated and stained with H&E [238]. High resolution pictures were taken with an Aperio ScanScope.

5.2.13.2 Immunodetection

Conditions for immunoblotting were as described previously [239]. The following antibodies were used: actin (sc-1616), p21 (sc-6246), Notch1 (sc-6014) (Santa Cruz), involucrin (Abcam, ab68) loricrin, filaggrin, Hes1 (AB5702) and CYFIP1 (07-531) (Millipore). Immunohistochemistry was performed as reported previously [240]. Briefly: 3- to 5-µm adjacent sections of formalin-fixed paraffin-embedded tissue arranged in a tissue microarray were used. The deparaffinized sections were heated in a 100-W household microwave oven at maximum power for three times 5 minutes each in 10 mmol/L citric acid for antigen retrieval. Primary antibody was applied for 16 hours at 4°C. Secondary staining was performed using the DAKO APAAP kit. The immunohistochemistry results were quantified by two independent persons. The signal intensity was graded into arbitrary units from 1 point which referred to no signal up to 10 points which referred to a very strong signal. The samples were analyzed for the total signal intensity that included the signal from all the epidermis layers.

5.2.14 Cloning

Construction of DNA plasmids and vectors was done using NEB's Gibson Assembly Kit following the manufacturer's protocol. In short, the target plasmid was cracked via restriction enzyme digestion, followed by size separation on a 1-2% agarose gel. The according bands were identified by UV illumination and cut out, followed by DNA cleanup and zipping in order to prevent religation. Synthesized DNA fragments had to be equipped with 20 – 30 nucleotide overhangs exactly matching the sequence next to the insertion sites within the destination plasmid. Digested plasmid and DNA fragments in a stochastic ratio of 1:3 had to be incubated together with Gibson Assembly Enzyme and Buffer at 37°C for one hour. Subsequently, the product could be transformed into competent cells on ice for 10 minutes followed by plating on agar plates and overnight 37°C incubation. Successfully ligated constructs gave rise to

bacterial colonies under antibiotic selection which were expanded in liquid LB broth followed by DNA plasmid extraction using mini/maxi prep kits.

5.2.14.1 Construction of Tet-On miRNA over expression plasmids

miR-181a hair pin sequence plus 210 base pairs of the flanking region in either direction and the according Gibson Assembly adapters were synthesized (Integrated DNA Technologies). The inserts were cloned into digested pTRIPZ (EcoRI and XhoI) using NEB's Gibson Assembly Kit and following manufacturer's protocol.

5.2.14.2 Generation of cell lines stably overexpressing KRAS

pUNO KRAS over expression plasmid (Invovogen) was transfected into cell lines as described above. Positive cells were selected using Blasticidine (5µM). Clones were picked, expanded and checked for stable KRAS expression via Western blotting. For *KRAS* 3'UTR experiments pGL4.75-KRAS LCS6m was used as a KRAS 3'UTR donor and sub cloned into pUNO KRAS with NheI-HF and T4 DNA ligase. Three miR-181a binding sites were mutated using Phusion-HF polymerase for mutagenesis and custom designed mutagenesis primers.

5.2.15 ChIP

Human epidermis was separated from the underlying dermis by a brief heat treatment and was minced finely in ice-cold PBS. Confluent primary keratinocytes as well as tissue samples were then cross-linked with 37% formaldehyde to a final concentration of 1% followed by the addition of glycine (final concentration 125 mM). After cross-linking, tissues were washed twice with 10 ml PBS with protease inhibitor. Tissue pellets were processed for ChIP assays as previously described using the rabbit anti-Notch1 antibody in parallel with affinity-purified non-immune IgGs [69]. Primers used for real-time PCR of the two regions of the human CYFIP1 promoter and for a region of human HES1 can be found in the materials section.

5.2.16 Luciferase activity assay

Human CYFIP1A promoter region was synthetized by Blue Heron Biotech (Bothell, WA) and inserted in pGL3-basic between the KpnI and BglII restriction sites (pGL3-CYFIP1A-3kb). SCC13

cells or primary keratinocytes were co-transfected with 0.5 µg of pGL3-CYFIP1A-3kb, 2 µg of the Notch1 expressing plasmid pcDNA3-Notch1 or empty vector as control and 0.05 µg of the Renilla internal control plasmid (phRL-TK). As a control of experiment, cells were also in parallel co transfected with 0.5 µg of the RBPjk-luc plasmid (Notch reporter plasmid), 2 µg of the Notch1 expressing plasmid pcDNA3-Notch1 or empty vector as control (pcDNA3) and 0.05 µg of phRL-TK. Cells were harvested 30 hours after transfection and assayed for Firefly and Renilla luciferase activity with the Dual Luciferase assay reporter kit. Results are expressed as relative firefly activity over Renilla luciferase activity. All experiments were performed in triplicates.

5.2.17 Invasion assay

Prior to the assay, cells were incubated for 48 hours at starving conditions with 10 x decreased concentration of supplements. The invasion capacity was assessed using 24 well-sized BioCoat Matrigel Invasion Chambers with 8 µm pore size. In order to hydrate the matrigel, inserts were incubated in basal cell medium for two hours at 37°C. Treated cells were suspended in basal medium at a concentration of 1 x10^5 cells /ml and 500 µl cell suspension was pipetted into the inserts. As a chemoattractant, wells of a 24 well plate were filled with 750 µl of full medium. After 24 hours, cells were either stained and used for the calculation of invasive capacity or mechanically detached from both sides of the PET membrane and assessed for protein expression by flow cytometry or Western blotting.

5.2.18 MEK inhibitor

For MEK inhibition GSK1120212 (Cellagen Technology, #C4112-5) was used at concentrations of 500 nmol/L. DMSO was used to prepare stock solutions and also as a control treatment during the experiments.

5.2.19 Statistics

All statistical evaluations were carried out using GraphPad Prism 5.0. The analyses were two-tailed Student's t-test of three independent experiments. Several experiments (except for *in vivo* experiments) were carried out three times independently followed by mean and error determination. The error bars represent standard deviations (SD). *In vivo* experiments were

evaluated using ANOVA with Bonferroni correction where error bars represent standard error of the mean (SEM). P-values of < 0.05 were considered significant.

6. Results

6.1 miR-181a decelerates proliferation in Cutaneous Squamous Cell Carcinoma by targeting the proto oncogene KRAS

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6.1.1 Abstract

Cutaneous squamous cell carcinoma (SCC) is the second most common human skin cancer with a rapidly increasing incidence among the Caucasian population. Among the many regulators, responsible for cancer progression and growth, microRNAs (miRNA) are generally accepted as key players by now. In our current study we found that microRNA-181a (miR-181a) shows low abundance in SCC compared to normal epidermal skin. *In vitro*, miRNA downregulation in normal primary keratinocytes induced increased proliferation, while *in vivo* miR-181a downregulation in HaCaT normal keratinocytes showed tumor-like growth increase up to 50%. Inversely, upregulation of these miRNAs in cancer cells lead to reduced cellular proliferation and induction of apoptosis *in vitro*. An in vivo therapeutic model with induced miR-181a expression in SCC13 cancer cells reduced tumor formation in mice by 80%. Modulation of miR-181a levels showed an inverse correlation with the proto oncogene *KRAS* both on mRNA and protein level by direct interaction. Knockdown of *KRAS* mimicked the anti-proliferative effects of miR-181a overexpression in patient-derived SCC cells and abolished the enhanced viability of HaCaT cells following miR-181a knockdown. Furthermore, phospho-*ERK* levels correlated with *KRAS* levels, suggesting that the observed effects were mediated via the MAPK signaling pathway. miR-181a seemed regulated during keratinocyte differentiation probably in order to amplify the tumor suppressive character of differentiation. Taken together, miR-181a plays a crucial tumor suppressive role in SCC by targeting *KRAS* and could be a promising candidate for a miRNA based therapy.

6.1.2 Introduction

Cutaneous squamous cell carcinoma (SCC) is the second most common skin malignancy in the general population with a rapidly rising incidence among Caucasians. It typically arises from intraepithelial lesions like actinic keratosis on sun-damaged skin [241, 242]. Disturbed differentiation represents a hallmark of SCC reflected by a diverse pattern of differentiation markers like Filaggrin and Involucrin or various keratins [243]. About 50% of all SCCs carry *p53* mutations and a typical UV signature presenting with cyclobutane pyrimidine dimers, making UV light a major SCC risk factor [244]. Organ transplant recipients (OTR), however, harbor a 65 – 250 fold increased SCC incidence compared to the general population due to continued immunosuppression [245]. Once SCC occurs, further SCC arising on sun-damaged skin are likely. Field-directed treatments like photodynamic therapy, imiquimod or ingenol mebutate are available, but all cause considerable inflammation and disfiguration [97, 115]. Here, future siRNA or microRNA based agents could prove beneficial by reverting a keratinocyte's course towards regular differentiation and cell death without inflammation.

miRNAs are approximately 20 nucleotide-long non-coding RNA molecules binding to the 3' untranslated regions (UTR) of target mRNAs in a sequence-specific manner influencing translation and/or stability of the transcripts [246]. miRNA effectively play roles in almost all aspects of cancer biology, such as in proliferation, apoptosis, metastasis and angiogenesis (reviewed in [247]). A large screen of miRNA expression in SCC singled out miR-181a as downregulated in our SCC patient samples. We thus studied expression and functionality of miR-181a in primary patient-derived and various cell lines representing normal skin and SCC. Our findings indicate a crucial role for miR-181a in regulating keratinocyte proliferation mediated by *KRAS* interaction and MAPK pathway inhibition, prompting us to validate these findings in a xenograft mouse model.

6.1.3 Results

miR-181a is downregulated in SCC compared to normal skin

In order to get a general overview into the miRNA landscape in SCC we extracted total RNA of the epidermal fraction of SCC from OTR (n = 8) and immunocompetent patients (n = 7) as well as normal skin control samples (n = 5).

The initial microRNA microarray screen revealed a highly deregulated miRNA pattern of the two SCC groups when compared to normal skin. miR-181a showed low abundance among SCC confirmed by TaqMan qPCR (Figure 1A). Notably, there was no difference in expression levels between OTR and immunocompetent SCC (data not shown).

Low levels of miR-181a result in increased cellular viability *in vivo* and *in vitro*

Decreased cellular presence of miRNAs presumably permits increased activity of their downstream targets and might in turn promote tumor formation. For the following functional assays, we set up a keratinocyte culture system from patient-derived SCC and normal cutaneous primary keratinocytes. In addition, we selected a panel of keratinocyte cell lines according to their basal miRNA expression levels (Suppl. S1). Since HaCaT, a cell line derived from normal human keratinocytes, and human SCC cell lines SCC13 and A431 exhibit miR-181a levels similar to patient samples, these cell lines were considered most suitable for our experiments.

Indeed, HaCaT cells harboring a stable miR-181a knockdown (kd miR-181a) and injected subcutaneously into nude mice had pronounced cyst formation capability while the respective control cells hardly formed a cyst (Figure 1B and Suppl. 3A and B). H&E histological sections of the kd miR-181a cysts did not only differ in size, but revealed pathological characteristics typical for intraepithelial SCC: Disturbed differentiation with distinct eosinophilic hyperkeratosis accompanied by parakeratosis (Figure 1C) akin to human SCC. Control cysts, on the other hand, showed relatively inconspicuous keratinocyte differentiation and basophilic cornification (Figure 1C). *In vitro* WST-1 assays confirmed the inverse correlation between cellular viability and miR-181a levels; kd miR-181a showed greater values compared to control cells (Figure 1D). HaCaT kd miR-181a cells isolated from cysts after the experiments endpoint or from *in vitro* cultures exhibited lower miR-181a levels when compared to control cells (Suppl. 3C and 4). On the contrary, knockdown of miR-181a did not induce proliferation

in primary SCC cells or SCC13 in which miR-181a was already lower, indicating that a 3-5-fold reduction in miR-181a was already sufficient to confer the maximum induction of proliferation (Figure 1F).

Due to its simplicity combined with a high degree of sensitivity as well as reliability, WST-1 is widely used to investigate differences in cellular proliferation rates. We are well aware that WST-1 is reduced in the mitochondria during events happening in the respiratory chain and reflects therefore metabolic activity. To evaluate this assay for our needs, we performed a WST-1 assay on healthy primary keratinocytes transfected with miR-181a or control inhibitors. In parallel all cells were counted at the starting and end point of the assay and normalized to control cells. In addition, we performed a BrdU incorporation assay, which represents another widely used, but more complex proliferation assay. Since the WST-1 measurement, manual cell counting and BrdU incorporation assay delivered comparable results, we concluded that mitochondrial activity adequately reflects cellular proliferation rates in keratinocytes (Suppl. 2). For the sake of correct scientific terminology however, we will stick to the term “viability” in our graphs.

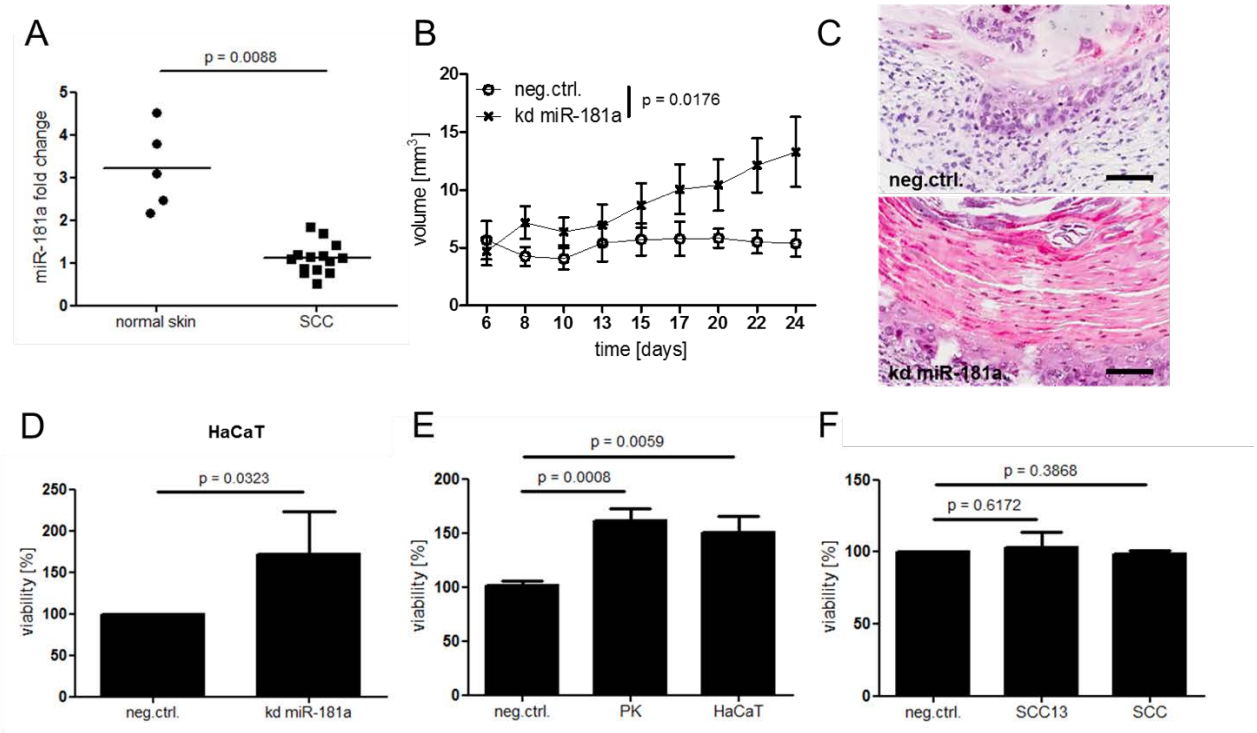


Figure 1: Low abundance of miR-181a is found in patient derived SCC specimen compared to normal skin and down regulation of miR-181a leads to increased viability in healthy keratinocytes

(A) Total RNA was isolated from patient derived SCC or normal skin biopsies (epidermal part). miR-181a levels were determined via TaqMan qPCR. (B) HaCaT knock down (kd) miR-181a or control cells

were injected subcutaneously into nude mice. Over all significance of the time course experiment was calculated using two way Anova and Bonferroni correction. A Student's t-Test was performed at the end point (day 24) (Suppl. 3B). (C) H&E sections of cysts. Black bar = 100µm. (D) Viability of HaCaT kd miR-181a cells *in vitro*. (E + F) Cells were transfected with miR-181a inhibitors for 48 hours. For *in vitro* viability assays cells were seeded into 96 well plates and incubated for 96 hours followed by WST-1 assay. P values for in vitro assays were Calculated via Student's T test. SCC = cultured SCC cells derived from patient samples, PK = cultured healthy keratinocytes derived from patient samples.

High levels of miR-181a result in decelerated cellular viability in vivo and in vitro

Following our observation of an inverse correlation between miR-181a levels and cellular viability, we were speculating that an upregulation of miR-181a would lead to decreased cellular viability in cancer cells. Therefore, we established a tetracycline-inducible miR-181a over expression model based on pTRIPZ Tet-On plasmid and SCC13 cells (Tet-On miR-181a). When injected subcutaneously into nude mice, control cells formed rapidly growing tumors, reaching termination criteria (tumor size > $1cm^3$ and/or ulceration) relatively early. miR-181a over expressing cells, on the other hand, grew slower and reach termination criteria at later time points (Figure 2A and Suppl. 5A - C).

In addition, Tet-On miR-181a showed decreased viability when doxycycline was added to the medium (Figure 2B). Maintained miR-181a expression in tumors with Tet-On miR-181a and in *vitro cell* cultures confirmed a robust miRNA induction by doxycycline activation (Suppl. 5D and Suppl. 6A). Fittingly, transfection of synthetic miRNAs (miRNA mimics) into cancer cells or stable miR-181a knock in led to decreased cellular viability accompanied by cell rounding and detachment (Figure 2D and supplementary 7B). FACS analyses as well as immunoblotting revealed a high number of apoptotic cells, identified by AnnexinV+/7AAD- staining and Caspase-3 cleavage respectively, compared to the control group transfected with control miRNA (Suppl. 7A). Interestingly, healthy primary keratinocytes and HaCaT cells were vulnerable to miR-181a upregulation to a certain degree as well (Figure 2E). An additional conformation was done in SCC13 cells transduced with a stable miR-181a over expression plasmid (ki miR-181a). As expected, these cells exhibited lower viability compared to control cells, while their miR-181a levels were strongly upregulated (Figure 2C and Suppl. 6B).

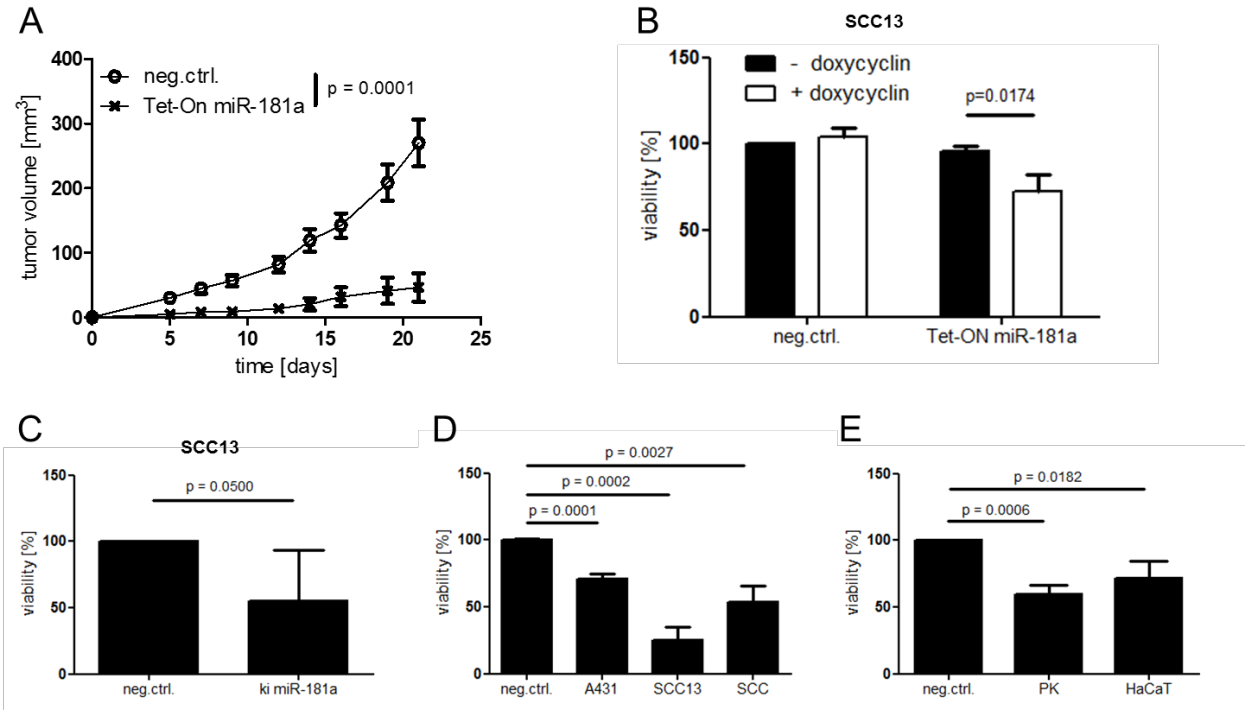


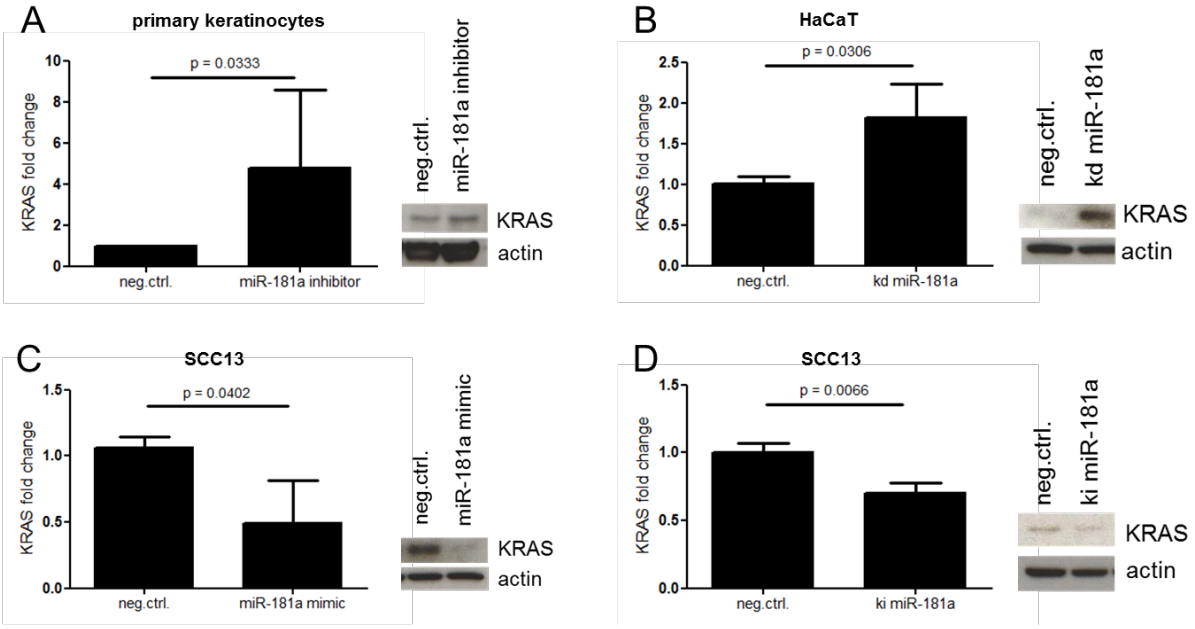
Figure 2: Up regulation of miR-181a leads to decreased viability in cancer cells

(A) SCC13 was transduced with pTRIPZ for inducible miRNA overexpression (SCC13 Tet-ON miR-181a) and injected subcutaneously into nude mice. Doxycycline (200 mg/kg) was administered via food pellets. Over all significance of the time course experiment was calculated using two way Anova and Bonferroni correction. (B) SCC13 Tet-On miR-181a were seeded into 96 well plates and exposed to doxycycline during the course of the experiment. Cells were transduced with pLKO.miRNA (ki miR-181a) for stable miRNA overexpression (C) or transfected with miRNA mimics for 48 hours (D + E). Cells were seeded into 96 well plates and incubated for 96 hours followed by WST-1 viability assay. P values for *in vitro* assays were calculated via Student's t-Test. SCC = cultured SCC cells derived from patient samples, PK = cultured healthy keratinocytes derived from patient samples.

KRAS is a direct target of miR-181a

Next we aimed to unravel the molecular mechanism laying behind miR-181a's negative effect on cellular viability. The proto-oncogene *KRAS* plays a critical role in a variety of malignancies and the interplay of miR-181a and *KRAS* has been described in other epithelial cancers [248]. Healthy cells with repressed miR-181a, either by transfection of miRNA inhibitors (Figure 3A) or by stable knock down (Figure 3B), showed higher *KRAS* protein and mRNA levels. SCC13 cells, on the other hand, exhibited lower *KRAS* levels when miR-181a was upregulated either by transfection of miRNA mimics (Figure 3C) or by stable miR-181a over expression (Figure 3D).

miRNAs are interacting with the 3'UTRs of their target mRNAs in a sequence-specific manner and are thereby interfering in the process of protein translation. In our approach we took advantage of the *KRAS* overexpression plasmid pUNO KRAS and cloned the according 3'UTR upstream of the start codon. A 3'UTR containing mutated miR-181a binding sites served as a control (Figure 3E). HaCaT cells stably transfected with these plasmids were characterized by elevated *KRAS* protein levels. However, only the cells containing the wild type *KRAS* 3'UTR were vulnerable to miR-181a mimic transfection resulting in reduced *KRAS* protein translation and thus in reduced cellular viability (Figure 3F). The same *KRAS* 3'UTR system transfected into HEK293T cells showed a similar picture. Cells harboring the wild type *KRAS* '3UTR had reduced *KRAS* protein after miR-181a mimics treatment while cells harboring a mutated 3'UTR were less responsive to miR-181a mimics (Suppl. 8).



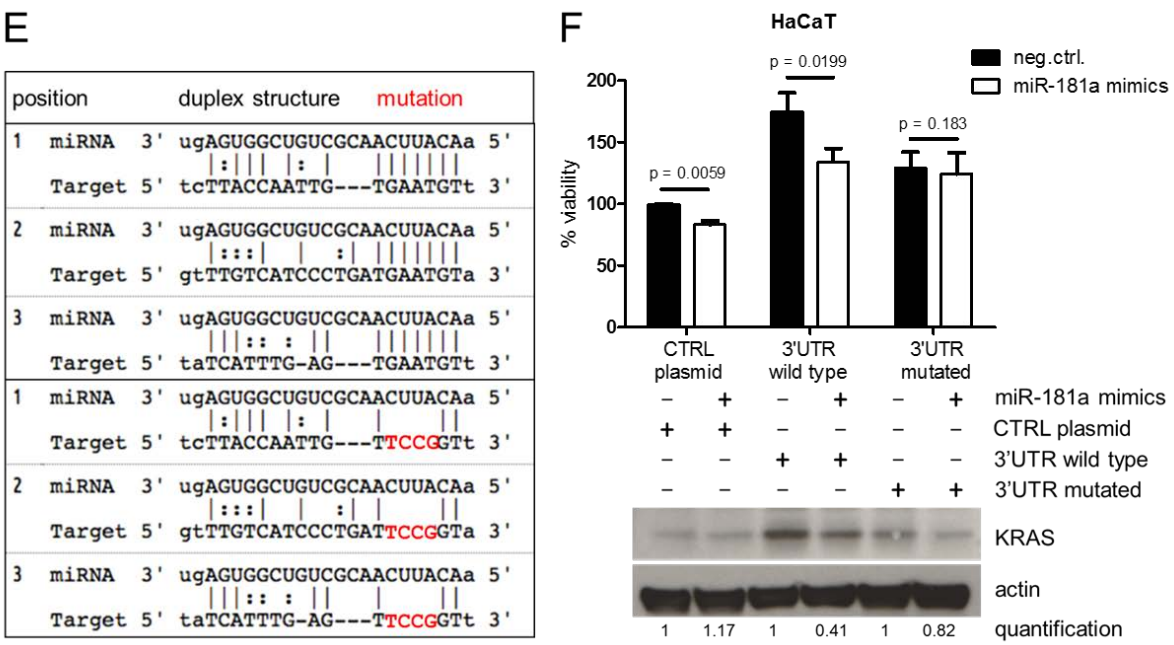


Figure 3: miR-181a targets KRAS directly

Cells were transfected with miRNA mimics (A) or inhibitors (C) for 48 hours or transduced with pLKO.miRNA.inhibitor / pLKO.miRNA (kd miR-181a / ki miR-181a) for stable miRNA modulation (B and D). Cells were lysed and mRNA or protein level analyses were performed using SYBR green qPCR or Western Blot respectively. (F) HaCaT cells were stably transfected with the indicated pUNO KRAS over expression plasmids in selection medium. Selected clones were transfected with miR-181a mimics for 48 hours following by WST-1 assay and Western Blot. (E) The panel in the left illustrates the three main miR-181a binding sites in *KRAS* 3'UTR and the according mutations. Student's t-Test was used to calculate P values.

miR-181a mediates its tumor-suppressive role through *KRAS* signaling via the MAPK pathway

Following the observation that miR-181a in keratinocytes correlated negatively with *KRAS* expression and with cellular viability, we were interested in a functional role of KRAS in keratinocytes. First we stably overexpressed *KRAS* in HaCaT using the pUNO *KRAS* plasmid (HaCaT pUNO KRAS) and performed a WST-1 assay. The result revealed that *KRAS*, when overexpressed, was able to boost viability in HaCaT similarly as a miR-181a knock down did. Notably, MAPK signaling was activated during this process (Figure 4A). Aiming to address the question whether there is a direct functional connection between miR-181a and *KRAS* we transfected HaCaT kd miR-181a with siRNA against *KRAS* or control siRNA. As expected, HaCaT cells harboring a stable miR-181a knock-down proliferated faster when only transfected with control siRNA, similarly as observed before. Reducing miR-181a's target *KRAS,* using siRNA, abolished this effect (Figure 4B). Notably, this effect was most likely not caused by siRNA off target effects, because two different siRNA sequences lead to similar results (data not shown). As mentioned before, MAPK signaling was activated as a result of *KRAS* overexpression. Therefore, we speculated that *KRAS* uses the MAPK signaling pathway to mediate its oncogenic signals in the cell. Initially, we treated HaCaT cells using epidermal growth factor (EGF) to trigger MAPK signaling, resulting in enhanced proliferation rates similarly as observed in HaCaT kd miR-181a (Figure 4C). Furthermore, we used HaCaT kd miR-181a which exhibited increased viability compared to control. This difference was abolished upon treatment with a *MEK* inhibitor (Figure 4D).

Suppression of *KRAS* by siRNA knock down, on the other hand, decreased viability in primary patient-derived SCC cells (SCC) similarly as miR-181a overexpression did (Figure 4E). Additionally, overexpressed *KRAS* rescued miR-181a mimics-induced effects in SCC13 to a large part (Figure 4F).

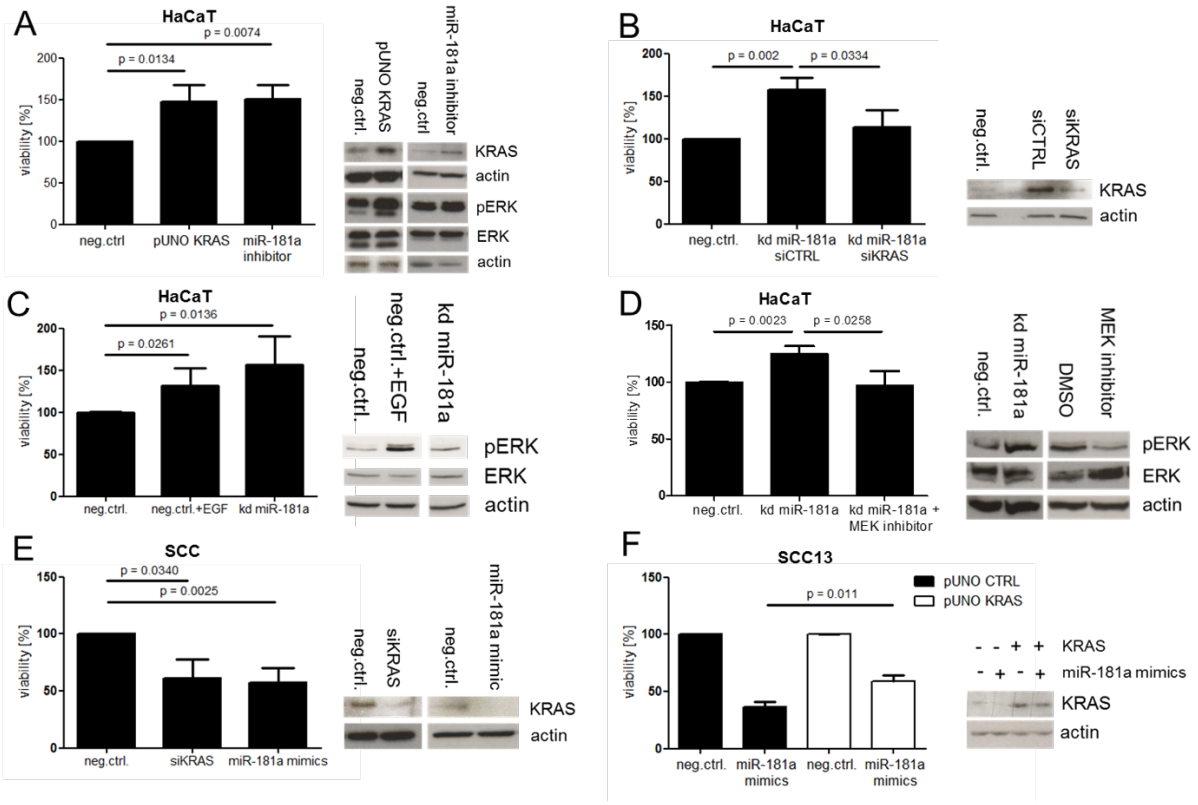


Figure 4: miR-181a mediates its tumor suppressive role through *KRAS* which signals via the MAPK pathway

Cells were transfected with the indicated siRNAs or miRNA mimics (A). In addition to siRNA/mimics the cells were transduced with the indicated plasmids (A - F). All transfections were carried out for 48 hours. A subset of the cells was seeded into 96 well plates, followed by WST-1 assay, while RNA and protein was isolated from the left overs. Protein and mRNA levels were determined by Western Blot or SYBR Green qPCR respectively. Student's t-Test was used to calculate P values. kd = knock down, SCC = cultured SCC cells derived from patient samples

miR-181a levels increased during keratinocyte differentiation

Since SCC can be characterized by disturbed differentiation, we speculated that miR-181a expression might be regulated during this process.

First we differentiated primary healthy keratinocytes by keeping them cultured confluent for a week. Direct cell-cell contact is required for *NOTCH1* activation which subsequently initiates the differentiation cascade. Comparing these matured cells with proliferating ones revealed increased miR-181a levels accompanied by clearly elevated differentiation markers in the differentiated subset (Suppl. 9A). SCC13 cells which cannot differentiate due to *NOTCH1* defects, regained the ability to mature when Notch1 was inducibly knocked in. During differentiation miR-181a levels went up (Suppl. 9C and Suppl. 10). Induction of keratinocyte

differentiation by adding Ca^{2+} to the medium or by UVA irradiation lead to similar results (Suppl. 9B + D).

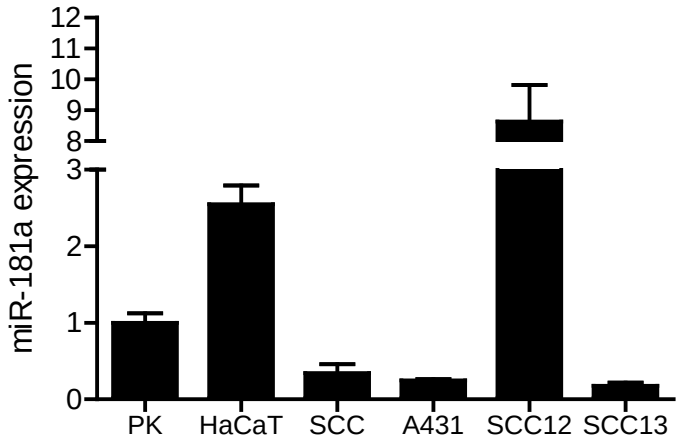
6.1.4 Discussion

In our study we have identified miR-181a as a critical factor in SCC progression. The published body of evidence on miR-181a mainly shows a carcinogenic role for miR-181a such as in [249-252], making miR-181a a so-called `oncomir´. A smaller part of the data on miR-181a, however, identifies miR-181a as a tumor suppressor in some cancers of the brain and the hematopoietic lineage [253-255]. This tumor suppression seems mainly to impact invasion and metastasis in tumors of the liver [256], salivary glands [257] and the ovary [258], and to a smaller part to impact proliferation such as in non-small cell lung cancer (NSCLC) [259] and acute myeloid leukemia [260]. These discrepancies on the function of miR-181a in different tumors, highlight miR-181a's context specificity depending on the environment. Our data show a tumor-suppressive role of miR-181a in the context of keratinocyte cancer with an impact on SCC proliferation. Contrary to miR-181a knockdown, reestablishing miR-181a levels in SCC attenuates cancer both *in vivo* and *in vitro*. Showing functionality beyond epithelial cancers, miR181a overexpression suppresses cell growth in xenograft tumors in chronic myelogenous leukemia [261] and large B-cell lymphoma [262]. The effect size on SCC in our study seems larger, suggesting therapeutic potential for miR-181a against SCC.

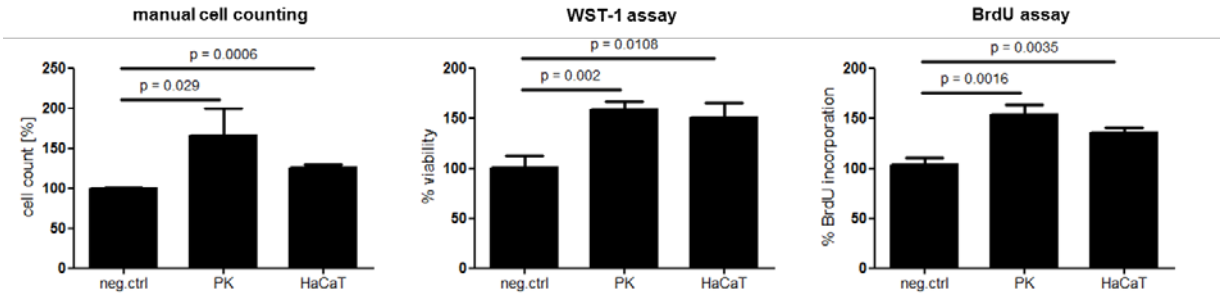
Based on known interaction of miR181a with *KRAS* [263], our study identified a direct interaction of the proto-oncogene *KRAS* with miR-181a in SCC confirming findings from other organ tumors [248, 259]. While this direct interaction has already been demonstrated using a dual luciferase assay [248, 260], we document this interaction in our system in a novel fashion by mutating the 3'-UTR of *KRAS* coupled with actual *KRAS* expression. This system allowed us to perceive the miR-181a-*KRAS* interaction above the background of endogenous *KRAS* expression. We thus support the previously found interaction between miR-181a and *KRAS* 3'UTR and directly link its main functional consequence to SCC, namely a change in cellular viability. The mechanism of *KRAS* in keratinocytes, however, is only sketchily described. Our data confirm that the well-known downstream mediators of *KRAS* function are found in the MAP kinase pathway [86, 257]. While *KRAS* acts as an oncogenic driver mainly by activating mutations [264-266], posttranscriptional control of *KRAS* by miRNA deregulation has been identified repeatedly [92, 93, 267, 268]. Our data suggest that such posttranscriptional control of KRAS by miR-181a may be critical in SCC development.

Differentiation is a key mechanism in cell control, maintaining homeostasis and preventing cancer development [60, 269]. Our data shows that differentiation in keratinocytes at large increases miR-181a expression, suggesting a role for miR-181a in a common pathway of differentiation, and that removing miR-181a is critical for the transition of keratinocytes into SCC. A similar weight has been reported in keratinocytes for miR-203 and miR-24 [270-272], underlining the impact of miRNAs in differentiation [273-275]. We thus believe that miR-181a is an important mediator of differentiation in keratinocytes. In summary, our data singles out miR-181a as a critical determinant in keratinocyte differentiation and control of SCC development. Manipulating miR-181a in vivo demonstrates the potential of miR-181a as a potential therapeutic miRNA in SCC.

6.1.5 Supplementary data

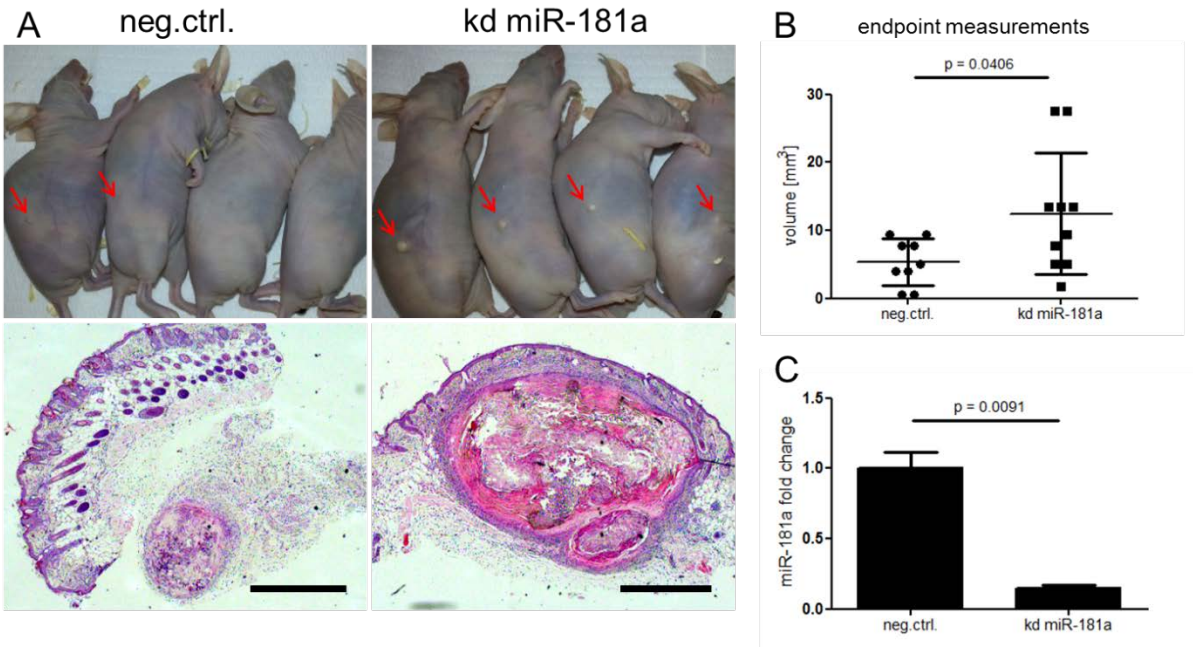


Supplementary Figure 1: Endogenous miR-181a levels of cell lines used in the present study
Total RNA was isolated and transcribed into cDNA. miR-181a levels of various cell lines were determined via TaqMan qPCR. PK = cultured healthy keratinocytes derived from patient samples, SCC = SCC cells derived from patient samples.



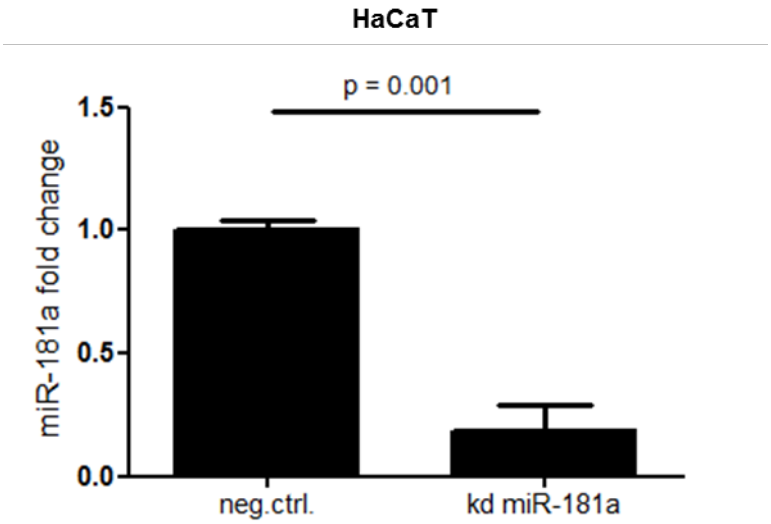
Supplementary Figure 2: WST-1 viability assay represents keratinocyte proliferation rates

Primary keratinocytes and HaCaT cells were transfected with miR-181a inhibitors or control sequence and seeded into Petri dishes. Cells were incubated for 96 hours and manually counted at the experiment's end point. WST-1 and BrdU assays were performed in 96 well plates after 96 hours of incubation time. Statistical analysis was performed using Student's t-Test. PK = cultured healthy keratinocytes derived from patient samples.

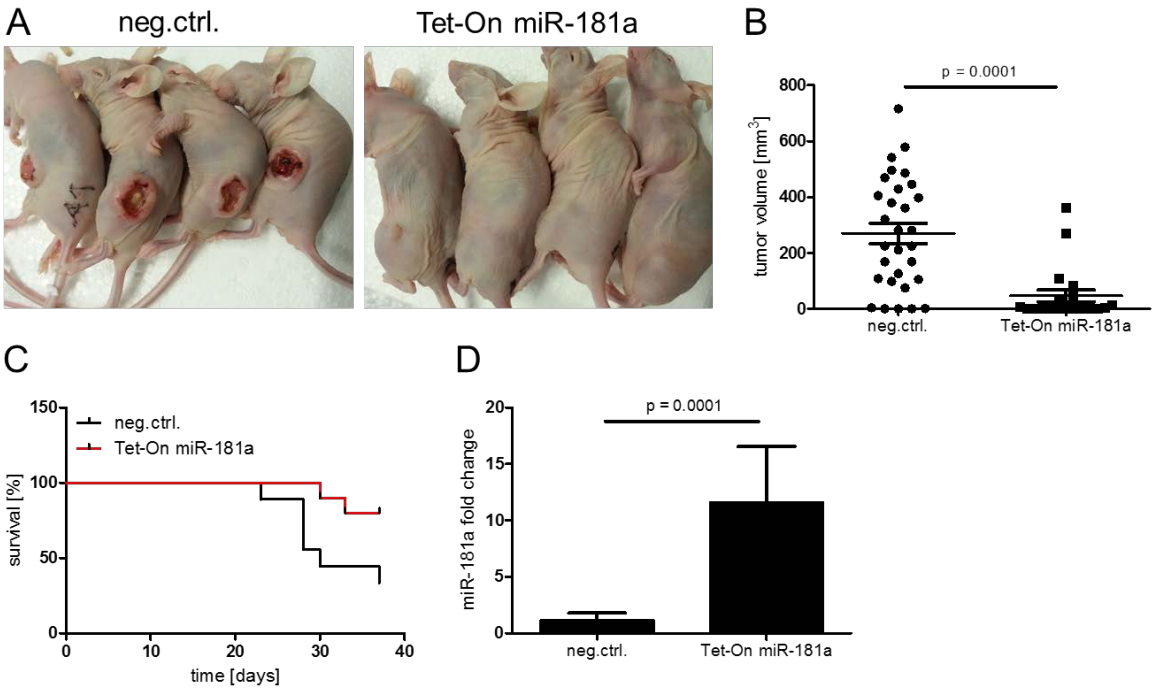


Supplementary Figure 3: miR-181a knock down promotes cyst formation *in vivo*

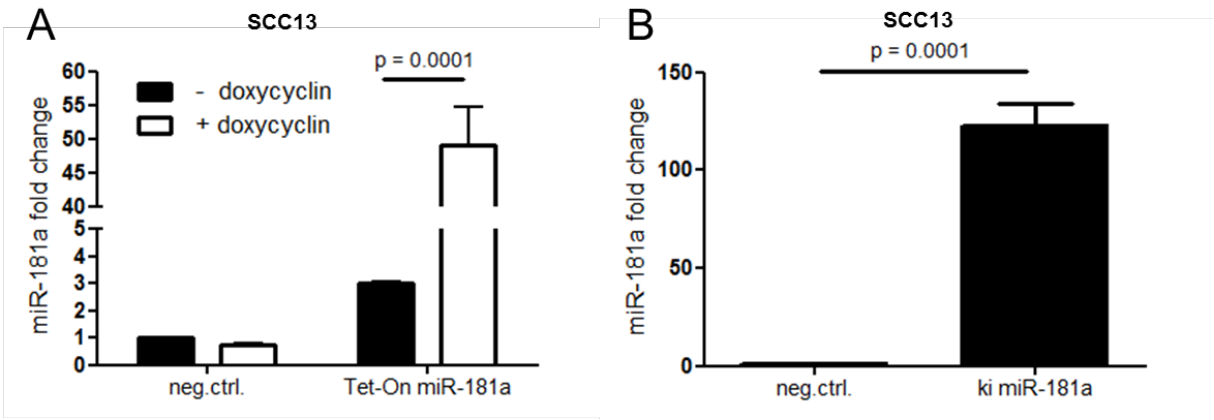
(A) HaCaT knock down (kd) miR-181a cells were injected subcutaneously into nude mice. Arrows in the upper panels highlight cysts or rudimental cysts. Lower panels show H&E stainings of the cysts. Length of black bars = 1mm. (B) Cyst volumes at the experiment's end point (day 24). (C) miR-181a levels of cysts isolated after mice were terminated. Whole RNA was isolated and transcribed into cDNA followed by TaqMan qPCR. Statistics were performed using Student's t-Test and Welch's correction. kd = knock down.



Supplementary Figure 4: miR-181a levels of HaCaT cells harboring stable miR-181a knock down. Whole RNA was isolated and transcribed into cDNA. miR-181a levels of HaCaT kd miR-181a were determined via TaqMan qPCR. Statistics were performed using Student's t-Test. kd = knock down.

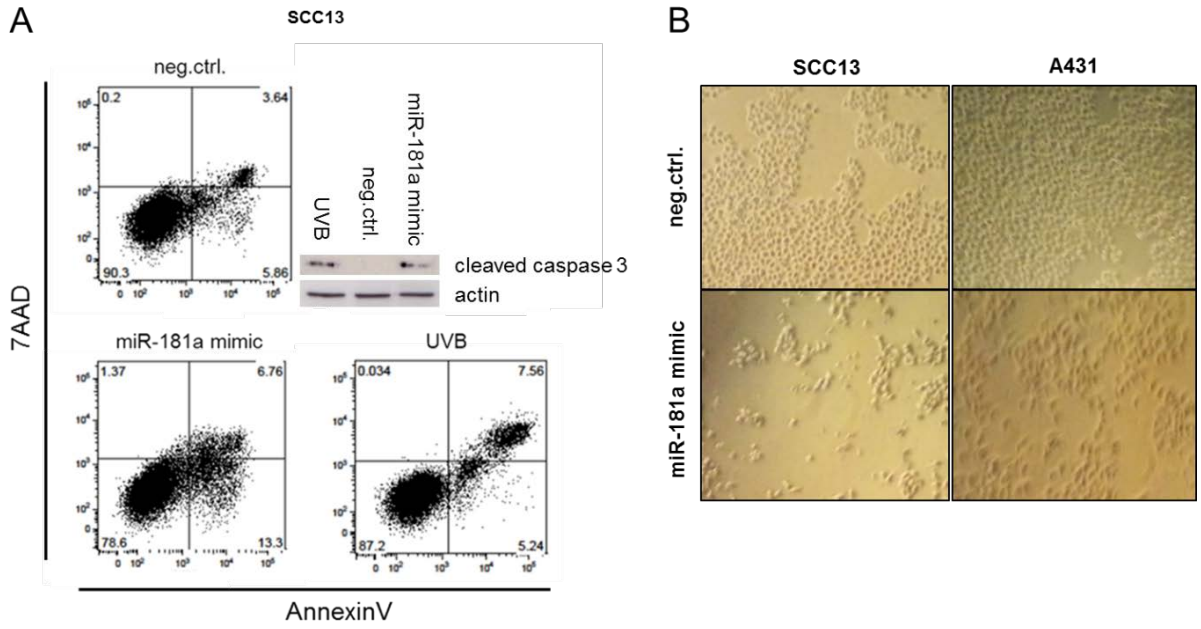


Supplementary Figure 5: Up regulation of miR-181a leads to decreased tumor growth in vivo
(A) SCC13 Tet-ON miR-181a were injected subcutaneously into nude mice. Doxycycline (200 mg/kg) was administered via food pellets. (B) Tumor volumes at the statistical endpoint of the experiment (day 21). (C) Kaplan-Meier survival curve. (D) miR-181a levels of tumors isolated after mice were terminated. Whole RNA was isolated and transcribed into cDNA follow by TaqMan qPCR. Statistics were performed using Student's t-Test and Welch's correction.



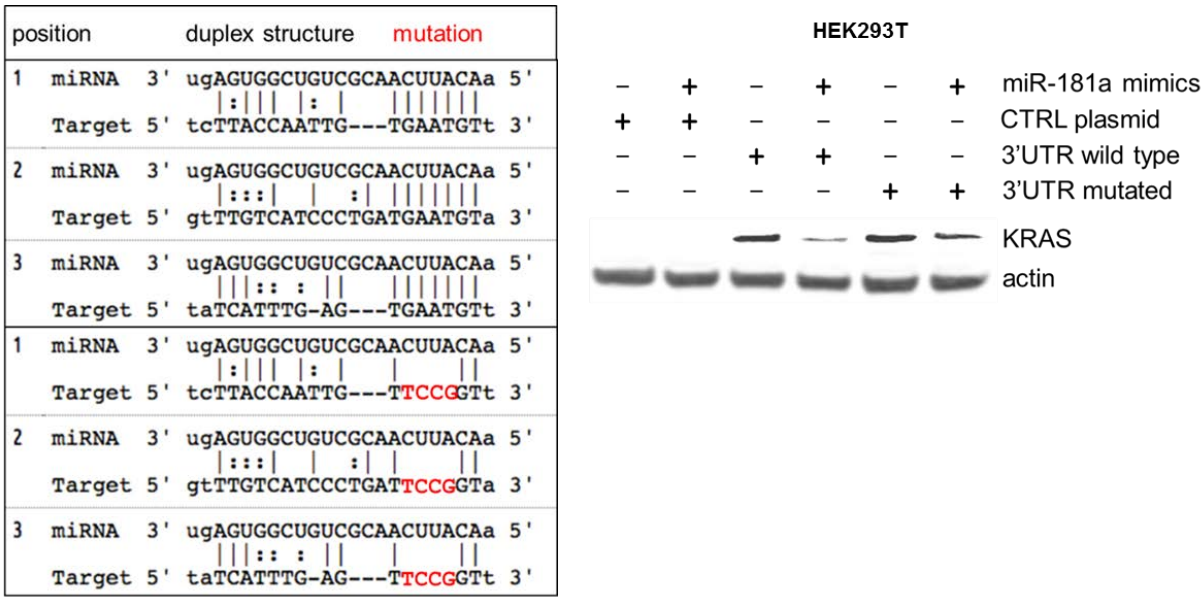
Supplementary Figure 6: miR-181a levels of SCC13 with artificial miR-181a up regulation.

(A) SCC13 Tet-On miR-181a were incubated with 500nM doxycycline for 48 hours. (B) SCC13 stably over expressing miR-181a. Whole RNA was isolated and transcribed into cDNA followed by TaqMan qPCR. Statistics were performed using Student's t-Test. ki = knock in.



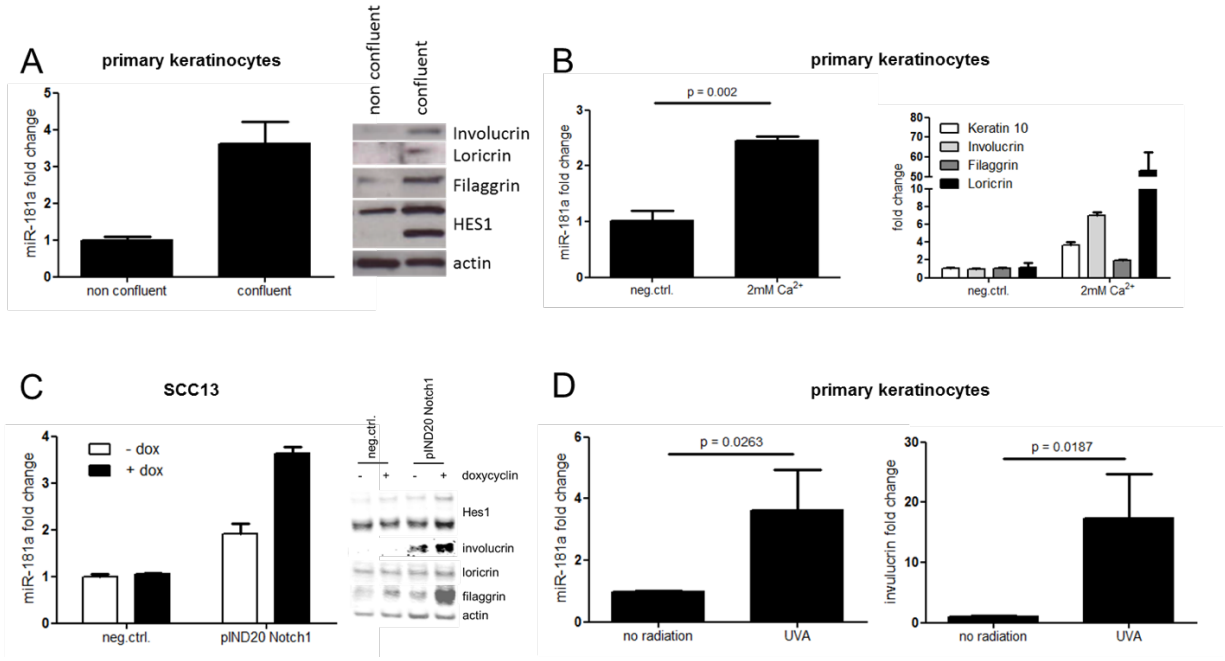
Supplementary Figure 7: miR-181a up regulation induces apoptosis in SCC13

(A) SCC13 cells were transfected with miR-181a mimics for 48 hours. One fraction was stained for 7AAD and AnnexinV following FACS analysis. Cells were irradiated with 0.06 J/cm^2 UVB for gating setup and served as a positive control. Protein from the other fraction was isolated and used for cleaved caspase 3 determination via Western blotting. (B). Cells were transfected with miR-181a mimics for 48 hours and seeded into petri dishes. 96 hours post transfection pictures were taken.



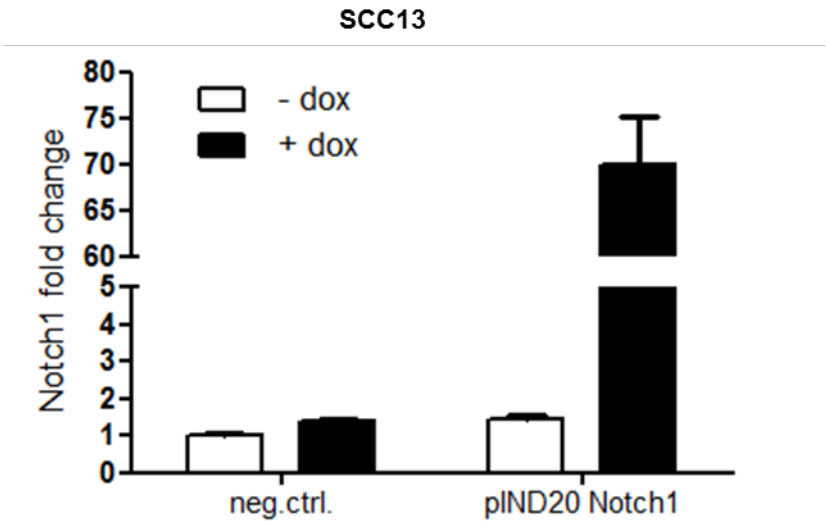
Supplementary Figure 8: miR-181a targets KRAS directly

Indicated pUNO KRAS over expression plasmids and miR-181a mimics were transfected simultaneously into HEK293T cells for 48 hours. Protein was extracted followed by Western Blot. The panel in the left illustrates the three main miR-181a binding sites in *KRAS* 3'UTR and the according mutations.



Supplementary Figure 9: miR-181a levels are increased during keratinocyte differentiation

Cells were differentiated by keeping them in a confluent state for a week (A), by Ca^{2+} exposure (B), by transducing an inducible *NOTCH1* plasmid (C) or by exposing them to 25 joule UVA irradiation. Protein and mRNA levels were determined by Western Blot or qPCR respectively. Student's t-Test was used to calculate P values.



Supplementary Figure 10: SCC13 cells harboring a tetracycline inducible Notch1 construct

SCC13 pIND20 Notch1 were incubated with 500nM doxycycline for 48 hours. Whole RNA was isolated and transcribed into cDNA followed by TaqMan qPCR. qPCR primers were designed to target the expression sequence (intra-cellular domain of Notch1) of pIND20 Notch1.

6.2 CYFIP1 is directly controlled by NOTCH1 and down-regulated in Cutaneous Squamous Cell Carcinoma

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Contribution to the manuscript:

As a shared first author, I contributed to this project by performing invasion assays as well as quantitative mRNA and protein analysis. Furthermore, I participated in experiment planning and discussion.

Running title: CYFIP1 in Cutaneous Squamous Cell Carcinoma

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6.2.1 Abstract

Squamous cell carcinoma of the skin (SCC) represents one of the most common cancers in the general population and is associated with a substantial risk of metastasis. Previous work uncovered the functional role of *CYFIP1* in epithelial tumors as an invasion inhibitor. It was down-regulated in some cancers and correlated with the metastatic properties of these malignant cells. We investigated its role and expression mechanisms in SCC.

We analyzed the expression of *CYFIP1* in patient derived SCC, primary keratinocytes and SCC cell lines, and correlated it to the differentiation and *NOTCH1* levels. We analyzed the effects of *NOTCH1* manipulation on *CYFIP1* expression and confirmed the biding of *NOTCH1* to the *CYFIP1* promoter.

CYFIP1 expression was down-regulated in SCC and correlated inversely with histological differentiation of tumors. As keratinocyte differentiation depends on Notch1 signaling, we investigated the influence of *NOTCH1* on *CYFIP1* expression. *CYFIP1* mRNA was highly increased in human *NOTCH1*-overexpressing keratinocytes. Further manipulation of the Notch1 pathway in keratinocytes impacted *CYFIP1* levels and chromatin immunoprecipitation assay confirmed the direct binding of *NOTCH1* transcription factor to the *CYFIP1* promoter.

CYFIP1 may be a link between loss of differentiation and invasive potential in malignant keratinocytes of cutaneous squamous cell carcinoma.

6.2.2 Introduction

Squamous cell carcinoma of the skin (SCC) belongs to the most common cancers in the world and it is the second most common skin malignancy in the general population [276]. It develops from atypical keratinocytes within sun-damaged epidermis, clinically visible as actinic keratosis or Bowen's disease, both considered non-invasive forms of SCC [277, 278]. Within the general population, about 1% of affected patients annually develop invasive SCC [37]. Unlike basal cell carcinoma – the most common skin malignancy - cutaneous squamous-cell carcinoma is associated with a substantial risk of metastasis [37]. The overall five-year rate of SCC metastasis is up to 5 percent [127, 279, 280]. The risk of recurrence or metastasis is related to the tumor size, location, depth of invasion as well as to histological differentiation [37, 280]. In the study by Rowe et al., poorly differentiated squamous cell carcinomas recurred at a rate of 28.6 percent and the five-year rate of cure after treatment was 61.5 percent, while in contrast well-differentiated tumors had a local-recurrence rate of 1.6 percent with a five-year rate of cure of 94.6 percent. In the study of Schmults et all tumor diameter of at least 2 cm, invasion beyond fat, poor differentiation, perineural invasion, and ear, temple, or anogenital location were risk factors associated with poor outcomes. Other studies have also shown that histological differentiation of tumors strongly correlates inversely with the metastasis rate, where poorly-differentiated SCC behaves most aggressively [281, 282].

Notch signaling is an important form of intracellular communication with a key role in cell-fate determination and differentiation [71]. In keratinocytes it induces differentiation and suppresses tumor development [283]. Its deletion in keratinocytes is sufficient to enhance susceptibility to skin cancer formation [239, 284] and loss of its dermal function contributes to field cancerization with development of intraepithelial and invasive SCC [16]. Notch1 is a trans-membrane receptor that is activated by ligand binding and proteolytic cleavage, with release of the intracellular domain [64]. The activated Notch cytoplasmic domain translocates to the nucleus, where it associates with the DNA-binding protein *CSL* and an ancillary protein, MamL1 or related family members [285, 286], forming a complex that is required for *CSL*-dependent transcription. Among others the best characterized targets of *NOTCH1* are *HES1*, *p21* and *IRF6* [68, 72, 287]. The molecular mechanisms downstream of Notch activation that elicit differentiation remain elusive.

Previous work of Silva et al. described *CYFIP1* as a novel putative invasion suppressor in a variety of epithelial cancers [147]. *CYFIP1* is a *RAC1*-interacting protein [288] which transmits signals from *RAC1* to the Arp2/3 complex by modulating the activity of the WASP family members, *WAVE1-3*, within the WAVE complex. WAVE-mediated activation of Arp2/3 induces the nucleation of G-actin to form a membrane protrusion, called lamellipodium, at the leading edges of cells growing in classical two-dimensional cultures [142, 289, 290]. It was shown that *CYFIP1* is commonly deleted in epithelial colon, breast or lung cancers. Reduced expression of *CYFIP1* was also observed during invasion of these tumors and was associated with a poor prognosis. *CYFIP1*-mediated depletion of *WAVE* function reduced epithelial adhesion and led to disorganization of tissue architecture [147].

In the present work, we show that *CYFIP1* is a direct *NOTCH1* target in keratinocytes. In this context Notch1 is an indirect inhibitor of cell invasion. These findings are of high clinical significance, as they suggest a rationale for the relationship between squamous cell carcinoma differentiation status and its invasive potential.

6.2.3 Results

CYFIP1 is down-regulated in cutaneous SCC

Decreased expression of *CYFIP1* has been reported in some cancers, such as colon breast or bladder cancer [147].The mRNA expression analysis in epidermis from cutaneous SCC samples showed reduced mRNA expression in tumors compared to the epidermis of normal skin (Fig 1a; normal epidermis: mean 0.011, SD ± 0.0059; SCC: mean 0.0069, SD ± 0.0027; p = 0.034). This difference was further confirmed by immunohistochemistry on 240 samples of SCC samples. *CYFIP1* expression was relatively high in normal skin, decreased in in-situ SCC and even more so in invasive tumors (Fig 1b, for representative staining see Fig 1c). Interestingly, *CYFIP1* expression was higher in the upper layers of the epidermis, typically layers with advanced differentiation, whereas the cells of the basal layer were mostly negative for *CYFIP1*. Since these basal cells show the lowest differentiation this finding suggests a relationship between keratinocyte differentiation and *CYFIP1* expression.

Further analysis of the invasive SCC demonstrated differential expression of *CYFIP1* after stratifying for histological differentiation status. Well-differentiated SCC showed a relatively high *CYFIP1* expression, which decreased in parallel with differentiation, i.e. with moderate expression in moderately differentiated tumors and low expression in poorly differentiated tumors (Fig 1d, for representative staining of the well, moderately and poorly differentiated SCCs see Fig 1e).

CYFIP1 expression was next assessed in cultured normal human keratinocytes (HKCs) and in established human squamous cell carcinoma cell lines derived from skin: SCC12, SCC13 and A431. Consistent with the analysis in human SCC samples, SCC12, SCC13 and A431 cell lines demonstrated a similarly decreased expression of *CYFIP1* on both mRNA as well as on protein level (Figure 1f and 1g). Consistent with the findings described above, the expression of *CYFIP1* as well as of the keratinocyte differentiation markers involucrin, filaggrin and loricrin increased in cultured HKCs upon induction of differentiation by growth to confluence. This up-regulation was observed at both the mRNA and protein levels (Figure 1h and 1i).

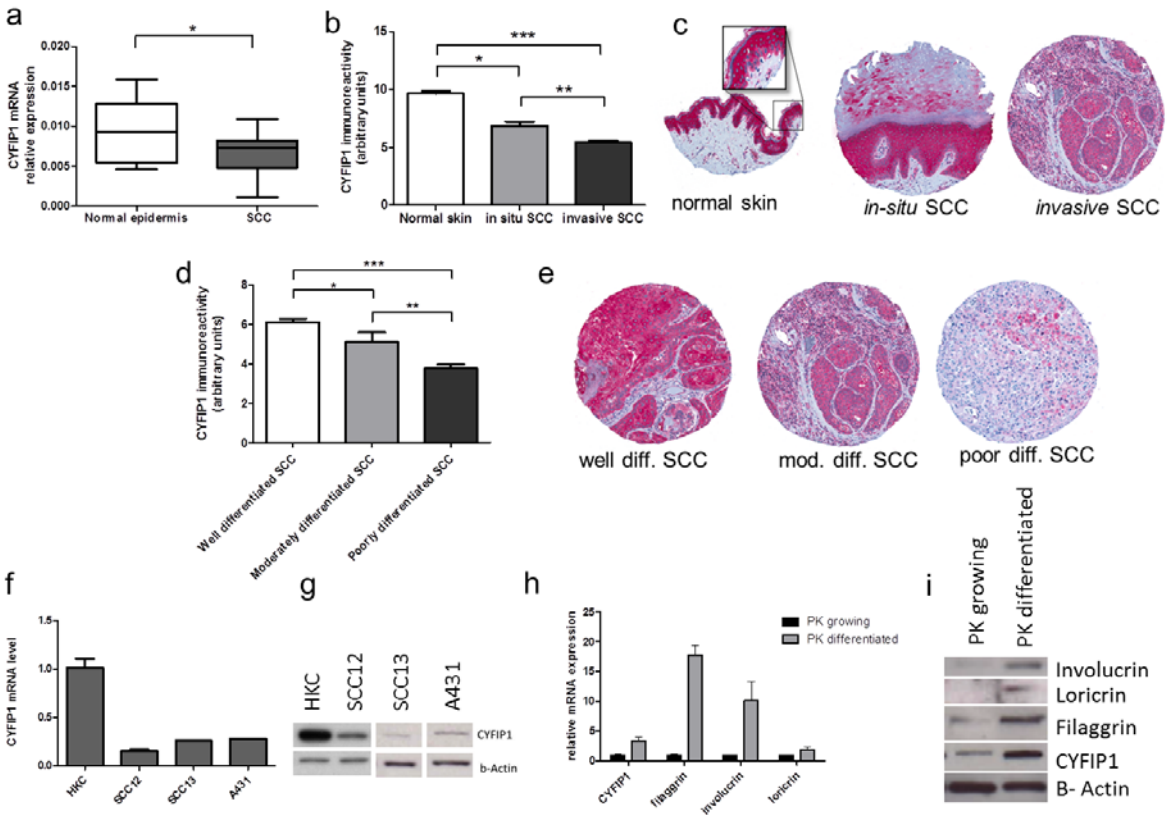

Fig 1. *CYFIP1* expression differs between normal keratinocytes and SCC cells. (a) *CYFIP1* mRNA expression was measured in epidermis derived from normal skin (n=9) and from patients SCC (n=30) samples. The RT-PCR demonstrated significant decrease of *CYFIP1* mRNA expression in the tumor samples *p<0.05. (b) *CYFIP1* protein expression in clinical samples was detected by immunohistochemistry which was performed on tissue microarray composed of 11 normal skin samples, 46 in-situ SCCs, and 240 invasive SCCs. The analysis revealed significant protein expression differences between all three groups of samples. Keratinocytes in the normal skin show high expression of *CYFIP1*, which is then decreased in in-situ SCC and even lower in invasive SCC *p<0.05, **p<0.01, ***p<0.001. The representative staining of normal skin, in-situ and invasive SCC are shown in the section c. (d) among the invasive SCC the expression levels of *CYFIP1* were related to the histological differentiation of the tumors. Well differentiated SCC showed relatively high expression of *CYFIP1*, which decreased with the loss of differentiation, so that the lowest expression was observed in poorly differentiated SCC. Figure 1e shows the representative staining of well, moderately and poorly differentiated SCC, *p<0.05, **p<0.01, ***p<0.001. (f) *CYFIP1* mRNA expression has been compared between normal human keratinocytes (HKC) and two squamous cell carcinoma cell lines: SCC12, SCC13 and A431. At the time of the experiment the cells were at about 70% of confluence. The *CYFIP1* mRNA levels were significantly decreased in the cancer cell lines as compared to normal keratinocytes. (g) Similarly to mRNA levels, *CYFIP1* protein levels were significantly lower in the cancer cell lines, as compared to normal human keratinocytes. *CYFIP1* expression in normal keratinocytes depends on their differentiation status. *CYFIP1* mRNA (h) and protein (i) are expressed in significantly higher amount in differentiated as compared to growing keratinocytes. Cells were at 50% confluence (growing cells), or

100% confluence for 4 days. After this time total RNA and protein were extracted and RT-PCR and WB performed respectively.

CYFIP1 gene expression is under direct positive Notch1 control in keratinocytes

Notch signaling promotes commitment of keratinocytes towards differentiation and thus prevents development of skin cancer [283]. The immunohistochemical analysis of human SCC showed coincidentally increased expression of the *NOTCH1* and *CYFIP1* proteins in upper layers of the epidermis, and differentiated keratinocytes (Figure 2a). The statistical analysis showed a moderate correlation between the immunoreactivity of these two proteins (correlation coefficient $r = 0.5$; $p < 0.0001$) (Figure 2b).

To assess whether *CYFIP1* expression is under the control of *NOTCH1* signaling pathway, SCC13 cells, previously reported as expressing low levels of *NOTCH1* [239], were stably transfected with a retrovirus overexpressing constitutive active form of *NOTCH1*. As shown in Figure 2c and 2d, expression of *NOTCH1* led to an induction of *CYFIP1* expression on both RNA and protein levels.

These results were further confirmed using SCC13 cells stably transduced with an inducible retroviral vector expressing a flag-tagged activated Notch1 protein fused to the human estrogen receptor (SCC13Nert). Conditional *NOTCH1* expression by 4-hydroxytamoxifen resulted in a substantial induction of *CYFIP1* (Fig 2e and 2g) in parallel with *HES1* (Figure 2f and 2g), a well-known direct target of *NOTCH1* [291].

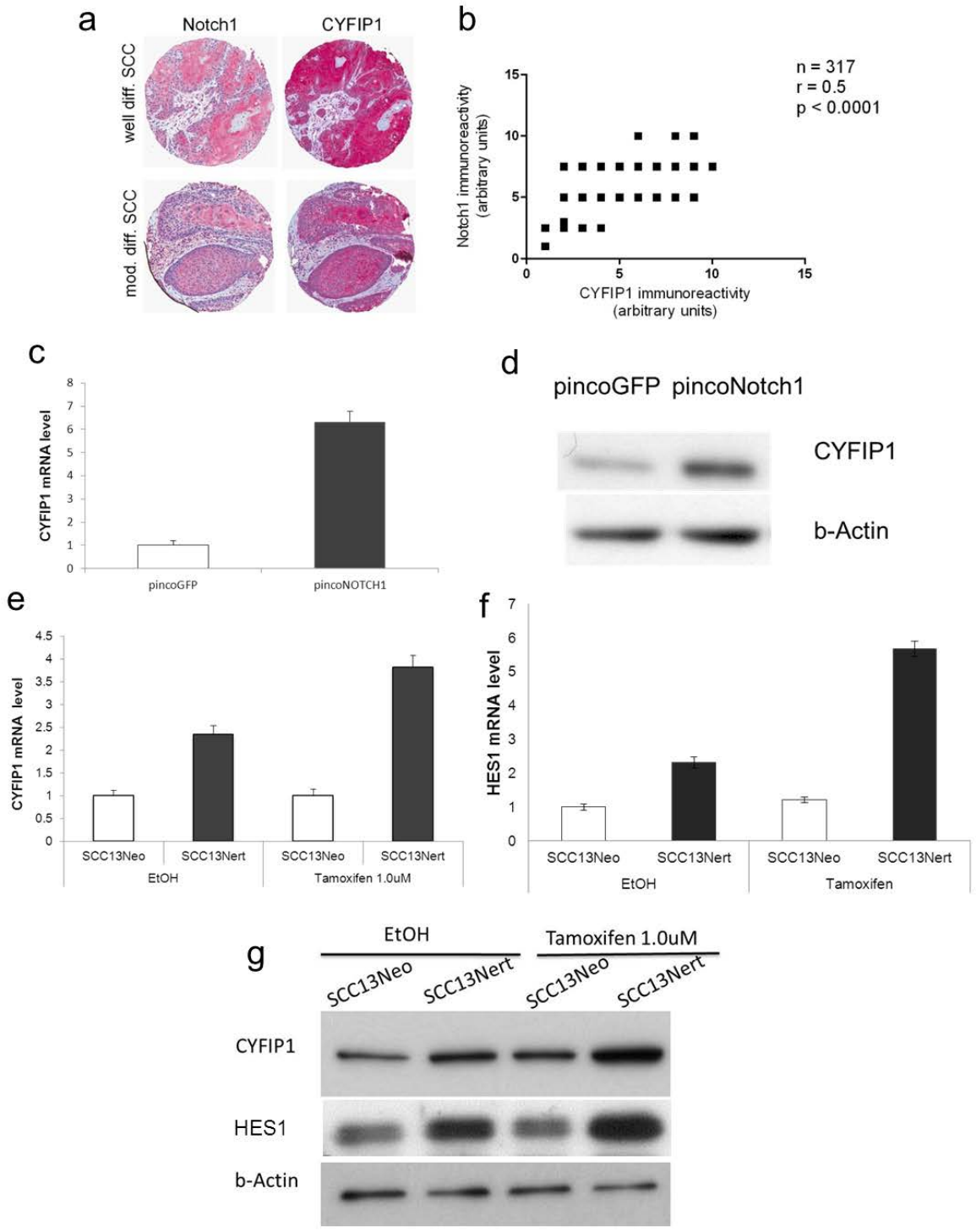

Fig 2. *CYFIP1* is induced during keratinocyte differentiation through a Notch-dependent mechanism.

(a) 4uM adjacent sections of SCC tissue microarray were separately stained for *CYFIP1* and *NOTCH1* (n=286). Immunohistochemical analysis of their expression in human SCC showed similar immunoreactivity patterns for both proteins. The expression was concomitantly increased in the suprabasal epidermis layers. (b) Statistical analysis of the immunoreactivity showed significant correlation of expression between *NOTCH1* and *CYFIP1* with the r = 0.5; p < 0.0001. (c) SCC13 cells were infected with a recombinant retrovirus expressing constitutively active Notch1 together with GFP

(pincoNotch1), or with a virus overexpressing GFP (pincoGFP) alone followed, 72h later, by mRNA and (d) protein expression analysis. Similar results were obtained in three independent experiments. (e) SCC13 cells were stably infected with a retroviral vector expressing a flag-tagged activated Notch1 protein fused to the human estrogen receptor (SCC13Nert), or empty vector control (SCC13Neo). Cells were subsequently treated with Tamoxifen at 1.0 uM concentration, collected after 30h and analyzed for *CYFIP1* and (f) *HES1* mRNA and (g) protein expression. Tamoxifen mediated activation of the Notch1 pathway significantly increased the mRNA and protein levels of both *CYFIP1* and *HES1* which has served as control downstream target.

Further sequence analysis of the proximal region of the human *CYFIP1* gene promoter revealed the presence of a "canonical" *CSL*-binding site located at -2.9kb and -0.9kb from the transcription start site (TSS) (Figure 3a). To verify *NOTCH1* binding to these sites, we performed ChIP assays with extracts from human primary keratinocytes under confluent, differentiating conditions and from normal human epidermis (Figure 3b and 3c respectively). ChIP analysis showed specific binding of the Notch1 protein to both of the predicted motifs within the promoter (-2.9 and -0.9 kb position).

Further functional analysis of the *NOTCH1* binding to the *CYFIP1* promoter was performed. Interestingly the overexpression of functional Notch1 did not increase the luciferase activity in the construct with the two putative *NOTCH1* binding sites of *CYFIP1* promoter (Figure 3d). To address the question of the *NOTCH1 – CYFIP1* interaction the cyclohexamide protein synthesis inhibition assay was performed (Figure 3e). SCC13 NeoNERT cells overexpressing Notch1 protein bound to the estrogen receptor were treated with cyclohexamide to block any further protein synthesis. With the addition of tamoxifen, preexisting *NOTCH1* was activated within the NeoNERT cells. This maneuver induced an increase in *CYFIP1* mRNA expression, proving the direct nature of the binding of Notch1 to the *CYFIP1* promoter.

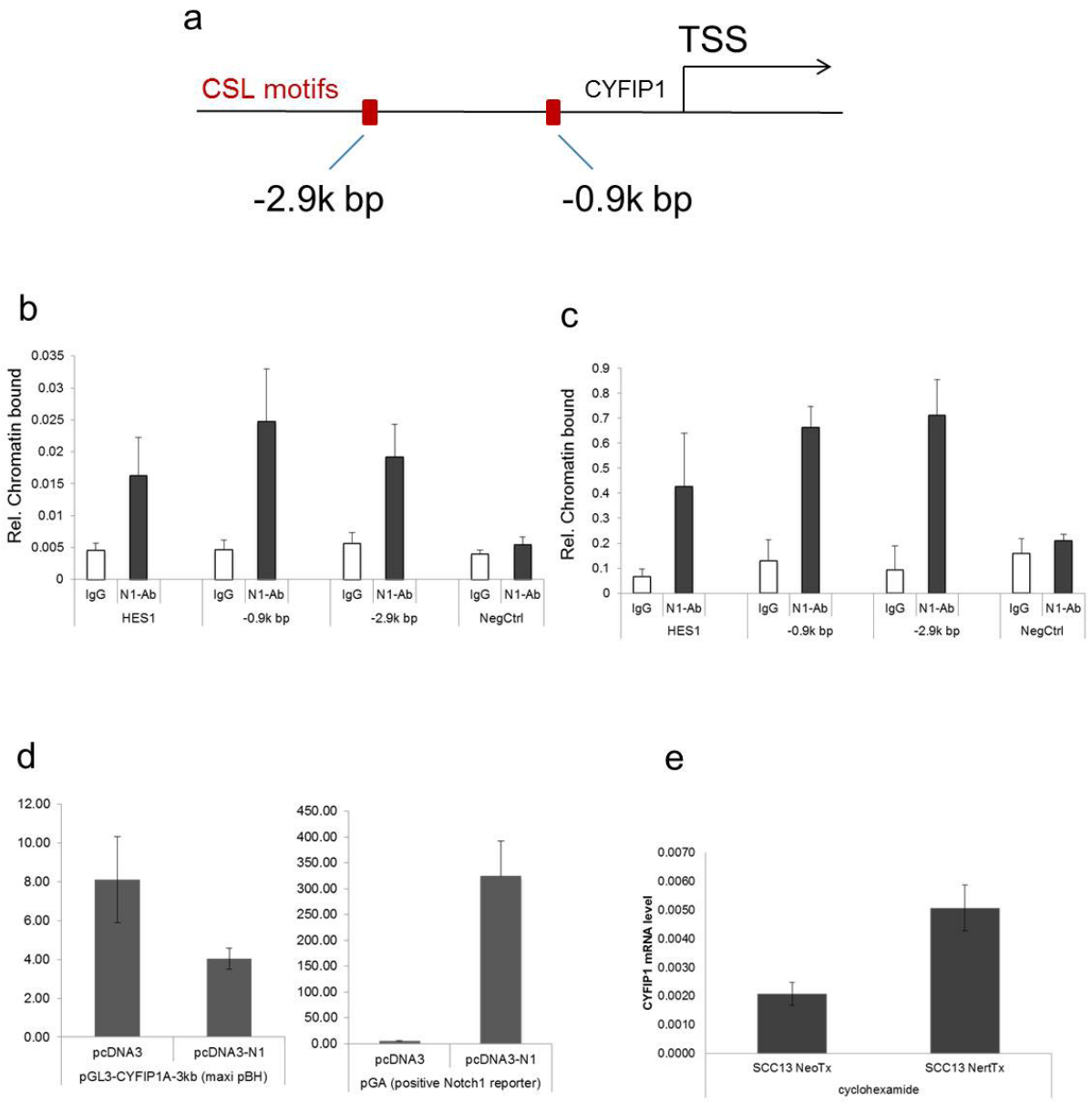

Figure 3. Endogenous Notch1 binds to the *CYFIP1* locus within specific regions of chromatin organization.

(a) Schematic illustration of ChIP results: TSS – transcription starting site, *CLS*-binding motifs are represented by the red bars. (b) Human primary keratinocytes and (c) total epidermis extracts of human epidermis were processed for ChIP assays using an antibody specific for *NOTCH1*, utilizing non-immune IgGs as control. PCR amplification of the various regions of the human CYFIP1 promoter encompassed the following CSL-binding sites: -2.9k bp: 5'-GAGGTGGGAACTA-3'; -0.9k bp: 5'-AATGTGAGAAAGT-3'. Un-precipitated chromatin preparations were similarly analyzed and used as "input DNA" control. The nucleotide sequence of the PCR primers is given in the materials and methods. The results are representative of two independent experiments. The relative amount of precipitated DNA, expressed in arbitrary units, was calculated after normalization for total input chromatin, according to the following formula [292]: % total = $2^{deltaCt}$ x 5 where detaCt = Ct (input) – Ct (immunoprecipitation). Ct, cycle threshold. (d) 3 kb *CYFIP1* promotor sequence with the two putative *NOTCH1* binding sites

was cloned and used for the luciferase activity assay. Increased luciferase activity was observed in response to *NOTCH1* overexpression in the positive control (pGA), but not in the cells with the cloned *CYFIP1* promotor. (e) To address the question of direct *CYFIP1* regulation by Notch1 a further experiment with the protein synthesis inhibitor – cyclohexamide was used. Briefly, NeoNERT cells overexpressing Notch1 protein bound to the estrogen receptor where treated with cycloheximide to block any further protein synthesis. With the addition of tamoxifen, preexisting *NOTCH1* was activated within the NeoNERT cells. This maneuver induced an increase in *CYFIP1* mRNA expression, proving the direct nature of the binding of Notch1 to the *CYFIP1* promoter.

Notch1 regulates cell invasion through *CYFIP1*

To verify the differential expression of *CYFIP1* between invasive and non-invasive cells, an invasion assay using a matrigel coated PET membrane was performed. This assay allows the distinction of invasive from non-invasive cells within the population of the SCC13 cell line. Flow cytometry showed a different distribution of *CYFIP1*-positive versus *CYFIP1*-negative cells in these two subpopulations (Figure 4a). Within the non-invasive population, 89.9 % of the cells brightly expressed *CYFIP1*, while only 7.52 % expressed *CYFIP1* dimly. Within the invasive population, however, most cells showed a dim *CYFIP1* expression, while only 23.4 % brightly expressed CYFIP1 ($p < 0.001$).

To verify Notch1 as a modulator of invasive capacity, SCC13 cells with Tamoxifen-inducible *NOTCH1* (SCC13Nert) were used in an invasion assay. To assess the interplay between *NOTCH1* activation, *CYFIP1* expression and invasion capacity, these cells were treated either with Cyfip1-specific siRNA (NertsiCYFIP1) or control siRNA (NertsiCTRL). Cells stably infected with empty vector control (SCC13Neo) and treated with *CYFIP1*-specific siRNA (NeosiCYFIP1) or control siRNA (NeosiCTRL) served as control for the Notch1 induction. Interestingly, the induction of Notch1 activity in SCC13 cells dramatically reduced their invasive potential (Figure 4b and 4c) in parallel to the induced expression of *CYFIP1* protein (Figure 4d). This reduced invasive phenotype was partially rescued by inhibition of Cyfip1. Induction of *NOTCH1* activity in the SCC13Nert cells was verified by the induction of p21 expression (Figure 4d). This experiment was repeated with two different *CYFIP1* specific siRNAs.

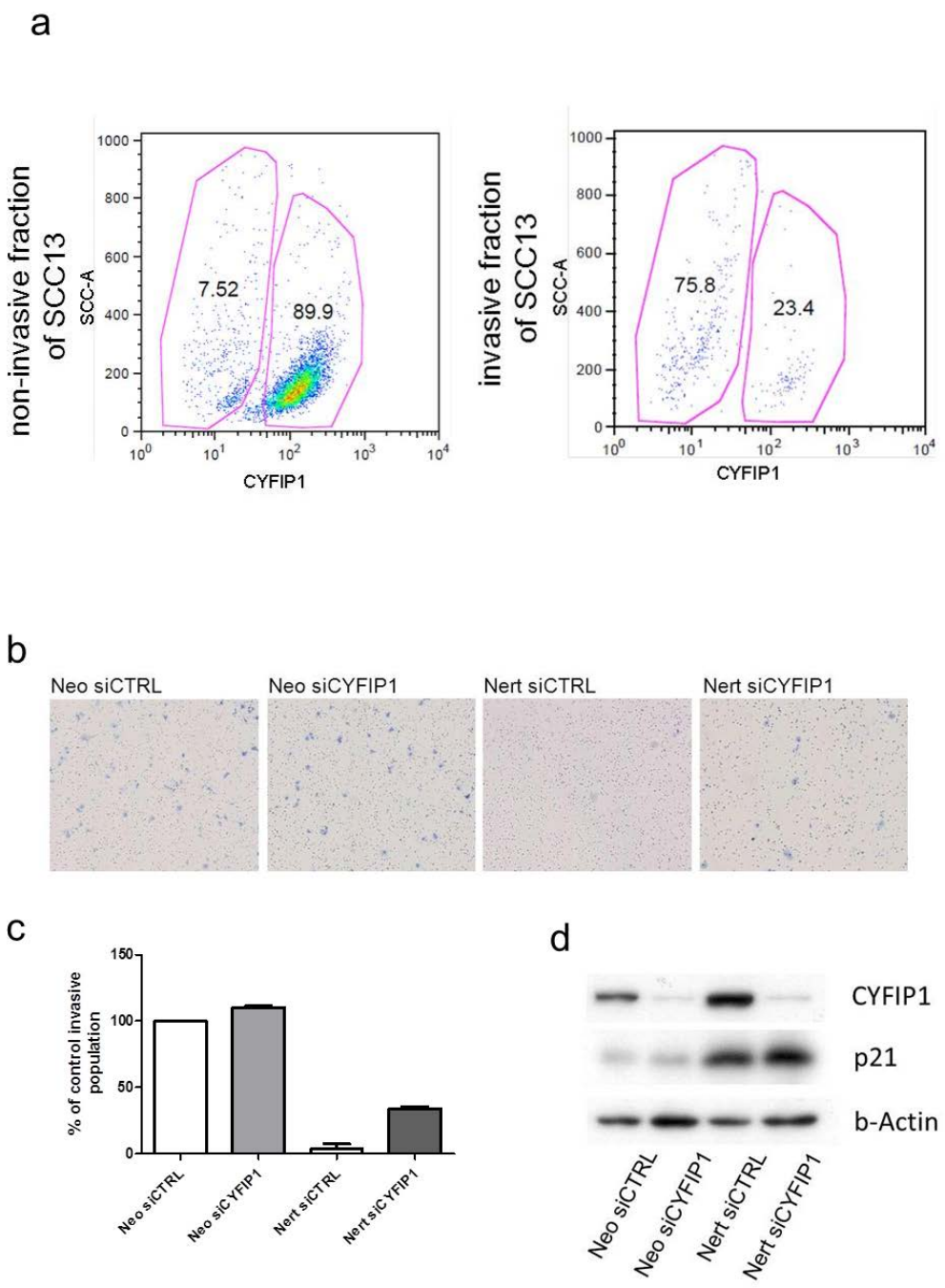

Figure 4. *NOTCH1* inhibits cell invasion through *CYFIP1*.

(a) SCC13 were starved for 2 days and then seeded in the matrigel coated chamber, where the coated membrane with 8 um pores separated the cells from the medium full in supplements. The cells were then cultured for 22 hours. After this timeframe the cells that migrated through the membrane were separated from the remaining cells and thus divided to invasive and non-invasive fraction of SCC13 cells. The cells were next stained for *CYFIP1* and analyzed by FACS. The invasive fraction of SCC13 cells shows a lower percentage of *CYFIP1* positive cells as compared to the non-invasive fraction ($p < 0.001$). (b) SCC13 cells were stably infected with a retroviral vector expressing a flag-tagged activated

NOTCH1 protein fused to the human estrogen receptor (SCC13Nert), or empty vector control (SCC13Neo). The cells were subsequently treated with siRNA targeting *CYFIP1* mRNA of unspecific control. The cells were next kept in starving conditions, seeded on the matrigel coated membrane with 8 um pores and treated with Tamoxifen. After 22h hours of incubation the invasive and non-invasive cells were counted and the invasion capacity was calculated as indicated in the manufacturer protocol. Section (b) represents the density of the invasive populations in the end of the experiment. (c) SCC13 with activated *NOTCH1* and treated with control siRNA (Nert siCtrl) showed drastically decreased invasive capacity, which could be partially restored when the Notch1 activated SCC13 cells were treated with *CYFIP1* specific siRNA (Nert siCYFIP1). (d) For control, the cells were parallel analyzed for *CYFIP1* and *p21* expression.

6.2.4 Discussion

The pro-differentiation and tumor suppressive functions of Notch signaling in keratinocytes are well established [283]. However, little is known about its mechanism. It has previously been shown that Notch activation is involved in in the cell-cycle control of keratinocytes via $p21^{WAF1/Cip1}$ [293]. Notch activation also induces differentiation of these cells through a more indirect mechanism, involving modulation of integrin expression in the basal layer, of *p63* as well as of IRF family members [72, 287]. We show here that Notch signaling is also involved in the regulation of keratinocyte invasive potential through the modulation of *CYFIP1* expression. To date, *CYFIP1* has been shown to inhibit tumor cell invasion in models of colon, lung and breast cancers [147]. It has been demonstrated that in these three particular tumors, its expression is decreased compared to corresponding normal tissues. In our study, *CYFIP1* expression was decreased at both the mRNA and protein level in in-situ and invasive SCC when compared to normal skin. Interestingly, progression of in-situ to invasive SCC was associated with a progressive downregulation of *CYFIP1* expression, suggesting a function for *CYFIP1* as an invasion inhibitor in SCC similar to that reported for other tumors. The histological differentiation of SCC is inversely linked to recurrence, invasion, and metastasis [37, 127, 280-282, 294]. Thus, poorly and undifferentiated SCC have the highest rate of invasion and metastasis, while moderately differentiated SCC were shown to have a higher rate of invasion and metastasis. Our analysis of *CYFIP1* expression in well-, moderately- and poorly-differentiated squamous cell carcinomas showed down-regulated *CYFIP1* expression in line with a loss of differentiation in tumor cells, suggesting a possible mechanism linking the loss of histological differentiation to increased invasive potential, a phenomenon observed also in other tumors such as esophagus, prostate, colon and breast [125, 295-298].

We show here that *CYFIP1* gene transcription is induced in differentiating keratinocytes by a Notch-dependent mechanism. Activation of Notch1 signaling, either directly by overexpression of an active Notch1 variant, or through a conditional manipulation, both increased *CYFIP1* mRNA and protein expression in cells. We further confirmed the binding of activated *NOTCH1* to the *CYFIP1* promoter within specific *CSL*-binding sites, revealing *CYFIP1* as a direct *NOTCH1* target. This binding was not confirmed by the luciferase assay, but further analysis with the cyclohexamide protein synthesis inhibition assay proved the direct nature of the binding of *NOTCH1* to the *CYFIP1* promotor. We also observed decreased levels of *CYFIP1* expression in SCC12 and SCC13 cell lines compared to normal human keratinocytes. This

decrease can be explained by compromised Notch signaling in these cells [239, 299]. These results explain the observed correlation in the expression of Cyfip1 and *NOTCH1* in the SCC tumors. Both *CYFIP1* and *NOTCH1* were relatively weakly expressed in the basal layer of the epidermis, which forms the leading front of the tumors. Both *CYFIP1* and *NOTCH1*, however, were upregulated in keratinocytes of the spinous and granular layer, e.g. in cells with increasing differentiation.

CYFIP1 expression has been negatively correlated with the invasion of malignant cells in lung, colon, breast and bladder tumors. We assessed whether these phenomena apply to cutaneous SCC as well. To distinguish migration from invasion, SCC13 keratinocytes were cultured on a matrigel-coated membrane. Under the starving conditions and chemotactic stimuli, keratinocytes are forced to inhibit differentiation and move in the direction of the attractant [300]. In line with the reported role of invasion suppressor for *CYFIP1*, the invasive fraction of SCC13 cells down-regulated their *CYFIP1* expression. Our results go along with the results of Silva and colleagues [147] where they also performed an *in vivo* assay where SCC cells with reduced *CYFIP1* expression showed drastically increased invasive properties Since *CYFIP1* is regulated by Notch signaling and plays the role in cancer cell invasion, the remaining question was if increased Notch signaling may influence this process as well. Our results revealed a drastic decrease in the invasion of SCC cells upon activation of the *NOTCH1* signaling pathway. This occurred in parallel with increased *CYFIP1* expression. Interestingly, however, the inhibition of *CYFIP1* expression in Notch-activated cells partially rescued the invasive capacity of the SCC cells. These results shed new light on the function of Notch signaling by suggesting *NOTCH1* to function as a promoter of differentiation and an inhibitor of invasion. The Notch1 mediated inhibition of invasion may be partially regulated by the *NOTCH1* mediated induction of *CYFIP1* expression. Our data are of likely clinical significance, because they suggest a mechanism for the observed correlation between the loss of tumor differentiation and increased destructive and invasive growth.

7. Discussion

The last chapter discusses the key finding of my PhD research work, puts them into a broader perspective and provides an outlook on possible future therapies. For more specific discussions dealing with the single projects please refer to the corresponding manuscripts in the results section.

Within the last decades SCC incidence has been constantly rising making SCC the second most common human skin cancer within the Caucasian population. Recent estimates on the show that about 2.1% of patients suffering from invasive SCC in the United States die [301]. Importantly, SCC does not necessary arise form KIN III AK, but has been shown to directly develop from KIN I and KIN II lesions as well. Since the cancer and its precursor lesions arise from body parts chronically exposed to solar UV light, sun protection is advised. Although, awareness of the danger posed by excessive UV exposure is generally given nowadays, the time lag between cutaneous sun damage and the consecutive SCC formation still results in increasing numbers of SCC cases. Due to the continuous intake of immunosuppressive drugs, OTR patients have a dramatically increased risk of developing SCC. Since the immune system plays a vital role in clearing critically UV damaged keratinocytes and therefore preventing SCC formation, OTR patients need continuous sun protection in the form of adequate clothing and high protection factor sun screen. Routinely, Invasive SCC is surgically excised without further ado. For AK and earlier stages of SCC there is a variety of topical treatments available which do result in reliable cancer clearance but induce disfiguring scaring through induction of inflammation and severe tissue damage. There is a high demand for alternative treatment strategies with potent inhibitory effects on cancer proliferation without causing excessive inflammation and leading to improved cosmetic outcomes.

In 1993 Ambros and colleagues discovered a novel non-coding class of small RNAs in *C. elegans*. Soon after, a huge research interest evolved around the newly discovered RNA molecules leading to the discovery of more and more miRNAs in various species including humans [302]. In the subsequent years researches were gradually understanding the impact of their findings on molecular Biology. It became evident that most of the genes are regulated

by miRNAs and that flaws within this complex network are associated with disease and even cancer.

Due to imperfect matching of the miRNA-mRNA complex, a miRNA typically targets many mRNAs and vice versa. Furthermore, the different expression levels of miRNAs and matching mRNAs between cells result in individual miRNA-mRNA target interactions with astonishing consequences. The depletion of a miRNA could lead to a cell cycle arrest in one tissue while it may induce uncontrolled proliferation in another [303, 304]. Although miRNA targets should be easily to predict by taking the rules of Watson-Crick into account, the actual situation is very different making context specific target identification and functional investigation inevitable [305].

Up to date, little is known about miRNAs in SCC.

A large microarray scan, performed by our collaboration partners in Lausanne, revealed a highly disturbed miRNA pattern in SCC. This circumstance is generally observed in cancer and was initially identified in B-cell chronic lymphocytic leukemia (CLL) [306]. Subsequent findings in patients with breast, lung, ovarian and cervical cancers underline the general prevalence of this phenomenon [307-309].

Among the miRNAs with low abundancy in SCC, miR-181a caught our attention due to its highly reproducible and even downregulation among patient samples (n=14) and most SCC cell lines. miR-181a seems to be affected in many cancers, such as ovarian, breast and colon cancer, suggesting devastating consequences of miR-181a deregulation in human organs. Indeed, miR-181a is not only a consequence of the chaotic transcriptome situation in cancer but a causative factor playing effective roles in the progression of these malignancies [258, 310].

The high context specificity, described above, seems to be particularly true for miR-181a [201]. The main body of cancer related miR-181a publications characterizes it as an oncomir with tumor promoting effects on cellular invasion and formation of metastasis. In a smaller subset of these studies miR-181a incudes cancerous proliferation, as demonstrated in gastric cancer [251]. Interestingly, miR-181a exhibits a tumor suppressive role in another subset of malignancies with consequences to various cancer hallmarks [256-259]. Along these lines, there is evidence form two studies suggesting that miR-181a may even overdue its anti-proliferative role, inducing autoimmunity, specifically systemic lupus erythematosus [311,

312]. Our results showed that modulation of miR-181a in SCC cells negatively correlated with cellular proliferation rates, making miR-181a a tumor suppressor in the context of keratinocyte carcinogenesis. mir-181a overexpression almost arrested SCC proliferation accompanied with induction of apoptosis, resulting in values down to 20% compared to the negative controls in our *in vitro* and *in vivo* experiments. miR-181a downregulation, on the other hand, accelerated proliferation in normal keratinocytes to an average of 150% accompanied with hyperkeratosis and parakeratoses, mimicking cancerous keratinocyte behavior. Showing functionality beyond epithelial cancers, miR181a overexpression suppresses cell growth in xenograft tumors in chronic myelogenous leukemia [27] and large B-cell lymphoma [28]. The effect size on SCC in our study seemed larger, suggesting therapeutic potential for miR-181a against SCC. Indeed, there is hope for patients suffering from skin diseases that they may profit from enormous efforts currently being undertaken to establish miRNA modulation as effective treatment any time soon. While most of the approaches focus on central organs, such as the heart, lung or liver, Guinea-Viniegra and colleagues successfully treated psoriasis by cutaneous application of miR-21 inhibitors in a mouse model [313].

Based on dual luciferase experiments performed in the context NSCLC, ovarian cancer and head and neck SCC, we chose to evaluate the impact of direct miR-181a - *KRAS* interaction in SCC [248, 259, 263]. Therefore, we modified the classical dual luciferase reporter assay using functional KRAS protein as a readout which simultaneously added another dimension to the approach. This system allowed us to perceive the miR-181a-*KRAS* interaction above the background of endogenous *KRAS* expression. We thus supported the previously found interaction between miR-181a and *KRAS* 3'UTR and directly linked its main functional consequence to SCC – increased cellular viability. Despite being the most frequently mutated oncogene in human cancer [314], little is known about *KRAS*' oncogenic significance in SCC so far. *KRAS* hyperactivity, caused by activating mutations as well as faulty posttranscriptional control, has been identified as a main tumor driver in cancers of the colon, pancreas and lung mainly mediated by MAPK signaling [92, 264, 266]. Our findings confirmed that the oncogenic axis of *KRAS* - *RAF–MEK–ERK* is controlled post-transcriptionally by miR-181a and may be critical in SCC development. Unsuccessful attempts in directly targeting Ras family members, by the use of small molecule inhibitors in the past, have characterized small GTPases as undruggable. Although, latest efforts in chemical structure determination reignited

researcher's hope, it will be a long and tedious way to a reliably functioning Ras inhibitor [314, 315]. Alternatively, miR-181a mimics could be adopted to tame *KRAS'* oncogenic features, at least in order to bridge the present times where no small molecule inhibitors are available.

Keratinocyte differentiation is a well-studied process in which the cells mature and eventually die. Along these lines, keratinocytes protect themselves from becoming cancerous by entering the differentiation cascade preponed. Exposure to carcinogenic factors like UV irradiation is a typical trigger for example [19]. During the keratinocyte differentiation process many tumor suppressive pathways are activated, leading to gradual metabolic shutdown and eventually to cell death [20]. Hence, keratinocyte differentiation is considered as a potential tumor-suppressive mechanism. Our data showed that differentiation in keratinocytes at large increases miR-181a expression, suggesting a role for miR-181a in a common pathway of differentiation, and that removing miR-181a is critical for the transition of keratinocytes into SCC. A similar weight has been reported in keratinocytes for miR-203 and miR-24 [40-42], underlining the impact of miRNAs in cellular differentiation [43-45]. We thus believe that miR-181a is an important mediator of differentiation in keratinocytes.

The highly conserved cell surface receptor Notch1 is known to initiate expression of various gene profiles, playing vital roles during human embryonic development and homeostasis of the adult body. Among Dermatologists *NOTCH1* is mainly known as a key trigger of keratinocytes differentiation and tumor suppression [283, 284, 316]. The exact mechanisms, however, are only sketchily understood. One piece was added to the puzzle by research performed in Gian-Paolo Dotto's lab where $p21^{WAF1/Cip1}$ was identified as a key mediator for Notch1 induced keratinocyte growth arrest [283]. More evidence underlining *NOTCH1*'s tumor suppressive role in keratinocytes was gathered by Proweller and Colleagues by constructing an *in vivo* mouse model expressing a dominant negative form of Notch1. As a result, these mice spontaneously developed SCC-like lesions on multiple body sites associated with Wnt/β-catenin accumulation in the nucleus [317]. In our recent study, we propose a novel mechanism of how *NOTCH1* suppresses another cancer hallmark – invasive potential, through *CYFIP1* upregulation.

In concert with a multi protein complex *CYFIP1* is known as a negative regulator of Arp2/3 induced actin remodeling, which is fundamental for keratinocyte migration and invasion [147, 318]. In models of colon, lung and breast cancers *CYFIP1* expression was decreased compared

to the according normal tissues and thus no longer able to inhibit cellular invasion [147]. Furthermore, histological data accessible via the "Human protein Atlas" reveals weak to moderate *CYFIP1* positivity in the majority of invasive cancers (http://www.proteinatlas.org/ENSG00000273749-CYFIP1/cancer, 2016-11-11) [319, 320]. In order to shed light on the *CYFIP1* situation in SCC, we conducted a comprehensive study, composed out of 286 SCC samples and corresponding normal skin. In accordance to the majority of other malignancies, *CYFIP1* expression was decreased at both mRNA and protein level in in-situ and invasive SCC when compared to normal skin. Furthermore, progression of in-situ to invasive SCC was associated with a progressive decline of *CYFIP1* histological positivity, suggesting a function for *CYFIP1* as an invasion inhibitor in SCC similar to that reported for other tumors [147]. Interestingly, we observed a correlation with the samples' differentiation state and *CYFIP1* presence. Numerous studies describe histological differentiation of SCC as being inversely linked to recurrence, invasion, and metastasis [4, 5, 7-9, 33][37, 127, 280-282, 294]. Well-, moderately- and poorly-differentiated squamous cell carcinomas showed decreased *CYFIP1* positivity in line with a loss of differentiation in tumor cells, suggesting a possible mechanism linking the loss of histological differentiation to increased invasive potential. A similar phenomenon was observed in tumors of the esophagus, prostate, colon and breast [125, 295-298].

In our study we report a mechanism in which Notch1 directly induced *CYFIP1* expression in differentiating keratinocytes. We achieved endogenous *NOTCH1* activation by culturing primary keratinocytes in a confluent state for prolonged times up to a week. The feasibility of this simple method is well known whereas successfully activated cells enter the differentiation cascade [60, 293, 321]. Alternatively, we artificially overexpressed *NOTCH1* in keratinocytes using different, well established, plasmid constructs [321, 322]. In either way, Notch1 expression correlated with *CYFIP1* expression on mRNA as well as on protein level. Along these lines we observed decreased levels of *CYFIP1* expression in SCC12 and SCC13 cell lines compared to normal human keratinocytes. This decline can be explained by jammed Notch signaling in these cells [239, 299]. Furthermore, we were able to confirm direct interaction of *NOTCH1* DNA interacting domain CSL with a putative *CYFIP1* promotor region via ChIP assay. For unknown reasons an actual transcription as a result of *CLS* binding to the *CYFIP1* promotor could not been observed via dual luciferase reporter assay. By choosing a cyclohexamide

protein synthesis inhibition assay instead, we were able to prove the direct nature of the binding of Notch1 to the *CYFIP1* promotor finally.

A negative regulatory role suppressing malignant invasion has been ascribed to *CYFIP1*, showing functionality in lung, colon, breast and bladder cancers [147]. We assessed whether this phenomenon applies to cutaneous SCC as well.

Another firm hint towards *CYFIP1* functionality as a negative regulator of cellular invasion was obtained by histological analysis of invasive SCC and normal human skin. Both *CYFIP1* and *NOTCH1*, were strongly expressed in keratinocytes of the spinous and granular layer, e.g. in cells with increasing differentiation. *CYFIP1* and *NOTCH1* protein, however, were hardly detectable in the basal epidermal layer, which forms the leading front of invasive tumors. Similar conclusions were drawn by histological observation of other invasive organ cancers [147].

To distinguish migration from invasion in an *in vitro* assay, SCC13 keratinocytes were cultured on a matrigel-coated membrane under serum starvation. FBS on the opposite side of the membrane forced them to inhibit differentiation and invade through the collagen gel layer [300]. Subsequent FACS analyses revealed lower *CYFIP1* levels of the invading cell fraction compared to the non-invasive part resting in the collagen layer. Silva and colleagues came to the same conclusion when performing an *in vivo* invasion assay where SCC cells with reduced *CYFIP1* expression showed drastically increased invasive behavior [147].

Having demonstrated that low levels of *NOTCH1* an *CYFIP1* are not only histologically associated with SCC but do functionally potentiate SCC invasiveness, the remaining question was whether this effect could be reverted by activating *NOTCH1* and the subsequent cascade. In another in vitro invasion assay, we stably overexpressed *NOTCH1* in SCC13 cells leading to *CYFIP1* expression accompanied by a complete abrogation of invasion. siRNA mediated *CYFIP1* knock down rescued the invasive nature of SCC13 cells. The effect size of *NOTCH1* induced abrogation of cellular invasion, suggests the Arp2/3 complex as a promising therapeutic target against invasive SCC. This speculation is supported by a comprehensive study in highly invasive pancreatic cancer, where Arp2/3 silencing tamed the cancers aggressive behavior [323].

To our knowledge, we are the first to report a functional mechanism connecting *NOTCH1* induced keratinocyte differentiation with *CYFIP1* mediated inhibition of SCC invasion. These

finding will shed new light on evaluating SCC differentiation status for diagnostic and prognostic purposes.

Overall the current PhD thesis contributes to a better understanding of the molecular mechanisms underlying critical SCC proliferation and invasion. Specific key players within this network, elaborated in our studies, may serve as potential starting points for novel SCC treatments. Together with our collaboration partners, we are currently investigating miRNAs and other factors specific to OTR. Our goal is to come up with new insights and treatment options against the SCC burden of these patients.

8. References

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9. Abbreviations

5-FU	5-Fluorouracil
80H-G	guanine to 8-hydroxyguanine
AK	Actinic keratosis
Ago	Argonaute
Arp2/3	Actin related protein2/3 complex
ATF3	Activating transcription factor 3
Blas	Blasticidin
BSA	Bovine serum albumin
BrdU	Bromodeoxyuridine
CD	Cluster of differentiation
CDKN2A	Cyclin-dependent kinase inhibitor 2A
C.elegans	*Caenorhabditis elegans*
ChIP	Chromatin Immunoprecipitation assay
CLL	Chronic lymphocytic leukemia
CLSM	Confocal laser scanning microscope
COX-2	Ccyclooxygenase-2
CsA	Cyclosporine A
CYFIP1	Cytoplasmic FMR1 interacting protein 1
DMEM	Dulbecco´s modified eagle medium
DNA	Desoxyribonucleic acid
Dox	Doxycycline
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EMT	Epithelial to mesenchymal transition
ERK	Extracellular regulated MAP kinase
FBS	Fetal bovine serum
FCS	Fetal calf serum
GAP	GTPase activating proteins
GDT	Guanosine diphosphate

GEF	GTP-exchange factor
GTP	Guanosine triphosphate
h	Hour
HCl	Hydrochloric acid
H&E	Hematoxylin and eosin
HIV	Human immune deficiency virus
HKS	normal human keratinocytes
HNSCC	Head and neck squamous cell carcinoma
HRAS	Harvey rat sarcoma viral oncogene homolog
IgG	Immunoglobulin G
IL	Interleukin
IL13RA2	Interleukin-13 receptor alpha 2
IL1R2	Interleukin-1 receptor 2
IM	Ingenol mebutate
JNK	Jun amino-terminal kinase
KIN	Keratinocyte intraepidermal neoplasia
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
MAPK	Mitogen-activated protein kinase
min	Minutes
miRNA	microRNA
miR-181a	microRNA-181a
mTOR	Mechanistic Target of Rapamycin
NFAT	Nuclear factor of activated T cells
NRAS	Neuroblastoma RAS viral oncogene homolog
NRF2	Nuclear factor erythroid 2-related factor 2
NSCLC	Non-small cell lung carcinoma
ORF	Open reading frame
OTR	Organ transplant recipient
P-Bodies	Processing bodies
PBS	Phosphate-buffered saline
PCR	polymerase chain reaction
PDT	Photodynamic therapy

PKC	Protein kinase C
PMSF	Phenylmethylsulfonylfluorid
Puro	Puromycin
RAF	v-Raf murine sarcoma viral oncogene homolog
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
RTK	Receptor tyrosine kinase
SD	Standard deviation
SDS	Sodium dodecyl sulfate
sec	Seconds
SEM	Standard error of the mean
snRNA	Small nuclear RNA
SPF	Sun protection factor
TBS	Tris-buffered saline
TERT	Telomerase reverse transcriptase
TGFβ	Transforming growth factor β
TLR	Toll-like receptor
USZ	University Hospital Zurich
UTR	Untranslated region
UV	Ultra-violet
WASP	Wiskott–Aldrich syndrome protein
WAVE	WASP family verprolin-homologous
WB	Western blot
Wt	Wild-type
XP	Xeroderma pigmentosum

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